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Clinical and Experimental

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PORPHYRIN CHROMOGENS OR PRECURSORS IN URINE, BLOOD, BILE, AND FECES

C. J. Watson, M.D., Ph.D., R. Pimenta de Mello, M.D.,* S. Schwartz, M.D., V. E. Hawkinson, B.S., and I. Bossenmaier, B.S.
Minneapolis, Minn.

THE occurrence of a colorless chromogen in the urine of patients with certain forms of porphyria has been recognized for many years. The term porphobilinogen was applied by Waldenström and Vahlquist[1] to a chromogen in these urines characterized by an Ehrlich aldehyde reaction and believed by them to be the precursor of the Waldenström uro-type porphyrin. Although it has been known that coproporphyrin (I or III) is readily reduced by sodium amalgam to a leuco compound,[2, 3] remarkably little attention has been given to the occurrence of this substance or of any chromogen or precursor of coproporphyrin in nature. Fischer, it is true, in his initial isolation of coproporphyrin from the famous porphyria case Petry, noted that it was present in the feces largely as a chromogen.[4] De Langen, in 1948,[5] refers briefly to the occurrence in nature of porphyrin leuco compounds and mentions the necessity of oxidation to permit detection. He states that he has not encountered porphyrinogens in blood serum but does not indicate in what materials they were encountered, nor what means of oxidation should be employed. There have been, in addition, a few brief references in the literature to the possible significance of a coproporphyrinogen.[6-10] In part these have been purely speculative and in part it was not clear that a chromogen of copro- rather than or together with uroporphyrin was present.[9]

Nevertheless, there have been two papers in which a chromogen of coproporphyrin was clearly recognized; that of Saillet[11] more than fifty years ago and that of Meek and co-workers in 1948.[12] The latter authors observed that the addition of hydrogen peroxide to certain urine samples caused an increase in fluorescence.

Saillet in 1896[11] stated that his "urospectrin" was excreted in considerable part as a colorless chromogen. Though any exact physical criteria are lacking, Saillet's descriptions leave little doubt that "urospectrin" was what is now designated as the urinary coproporphyrin. Although acquainted with this paper for a number of years, it is now quite evident that we as well as a numerous company of porphyrin investigators since Saillet have given insufficient attention to his observations. In fact, it appears to have been rather generally assumed that whatever fraction of the urinary coproporphyrin was

From the Department of Medicine, University of Minnesota Hospital.

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*Fellow of the Guggenheim Foundation. Present address: Oswaldo Cruz Institute, Rio de Janeiro, Brazil.

excreted as a chromogen was rapidly converted to the pigment, during its extraction and subsequent treatment. The reasons for this faulty assumption have now become evident and will be considered in the following.

Our own search for a chromogen or precursor of coproporphyrin was stimulated principally by two factors: (1) the observation that in experimental animals increases of coproporphyrin in urine or blood serum were observed under certain conditions such as ultraviolet radiation or photodynamic activity,* and (2) the recovery of greater amounts of urinary coproporphyrin by a new quantitative procedure than obtained by the modification[13] of Fikentscher's method[14] which has been used in this laboratory for several years. The purpose of the present communication is to describe certain properties of the urinary chromogen of coproporphyrin and to consider its significance in relation to the total excretion of urinary porphyrin.

EXPERIMENTAL

The new method of determining urinary coproporphyrin is given in detail in the companion paper.[15] It will suffice to say now that it depends on a more sensitive fluorophotometer permitting utilization of but 5 ml. of urine which is extracted once with 100 ml. of ethyl acetate after addition of 5 ml. of buffered acetic acid. (Glacial acetic acid 4: saturated aqueous sodium acetate solution 1.) After washing the ethyl acetate with a 1 per cent sodium acetate and 0.005 per cent iodine solution, the coproporphyrin is extracted with 1.5N HCl and its concentration is determined by fluorimetry. In the present paper this method, with omission of the iodine from the washing solution, is designated as procedure II, procedure I being the "old" method involving ether extraction of 100 ml. of urine. It soon became apparent that the results with II were considerably higher than those with I. While it has been established now that the differences between the two procedures are explained on the basis of several factors, of which the chromogen is but one, a considerable study was required before the importance of the chromogen was realized. For a time the possibility was considered that the disparity might be due, at least in part, to an ethyl acetate-soluble, ether-insoluble uro-type porphyrin, or to a "complex" such as that recently described by De Langen,[5] and these were carefully sought, but not found. However, the following phenomenon clearly indicated the importance of a precursor or chromogen. If the primary ether extract, as obtained with procedure I[13] from relatively fresh urine, was extracted repeatedly with 1.5N HCl, until the latter, as well as the ether, no longer exhibited any red fluorescence in ultraviolet light, it was observed that after two to three minutes of radiation of the ether with near ultraviolet light from a 4H mercury lamp a considerable additional amount of coproporphyrin usually appeared. This increment did not constitute the entire difference between the two methods, and it was found, furthermore, that similar radiation of the ethyl acetate in procedure II was also productive of still additional amounts of porphyrin. Representative data are given in Table I.

*Unpublished observations.

TABLE I. ADDITIONAL AMOUNTS OF COPROPORPHYRIN OBSERVED IN 24-HOUR COLLECTIONS OF URINE AFTER ULTRAVIOLET IRRADIATION OF ETHER OR ETHYL ACETATE WITH PROCEDURES I AND II

CASE AND DIAGNOSIS	I		II	
	AMOUNT WITHOUT RADIATION (γ/DAY)	ADDITIONAL AMOUNT AFTER RADIATION OF ETHER (γ/DAY)	AMOUNT WITHOUT RADIATION (γ/DAY)	ADDITIONAL AMOUNT AFTER RADIATION OF ETHYL ACETATE (γ/DAY)
1. Poliomyelitis	89	26	133	25
2. Poliomyelitis	125	25	142	44
3. Poliomyelitis	57	41	155	12
4. Carcinoma of esophagus	99	9	152	
5. Hodgkin's disease	264	20	383	
6. Retroperitoneal sarcoma	72	10	137	53
7. Obst. jaundice	229	16	251	
8. Lead poisoning	567	19		
	646	3		
	721	5		
9. Hepatitis	157	57		
	180	116		
	158	124		
10. Porphyria	128	85		
11. Cirrhosis	178	8		
	184	61		
	149	34	213	
12. Hepatitis	284	20	383	

Pure coproporphyrin (type I) dissolved in N/40 NaOH is easily reduced with 3 per cent sodium amalgam. After shaking for but three minutes the solution becomes colorless and the red fluorescence in ultraviolet light becomes very weak. If this solution is radiated briefly in ultraviolet light, or exposed for a longer period to sunlight, the coproporphyrinogen is partially converted to coproporphyrin. Incomplete recovery of porphyrin occurs with either type of irradiation. As shown in Table II addition of coproporphyrinogen to urine resulted in an average recovery of only 44 per cent when procedure I and ultraviolet irradiation were employed. In this connection it is clear that certain oxidants or dehydrogenators, such as lead tetra-acetate or permanganate, are damaging to the porphyrin so that conversion of chromogen to porphyrin is far from quantitative. We have found the effect of H_2O_2 very inconstant and unreliable. It appears, however, that iodine in low concentration is quite

TABLE II. RECOVERY OF TOTAL COPROPORPHYRIN AFTER ADDITION OF PURE CHROMOGEN TO HUMAN URINE SAMPLES

SAMPLE	VALUES BEFORE ADDITION			γ ADDED	VALUES AFTER ADDITION*			RECOVERY (%)
	γ BY PROC. I	γ BY IRRAD. ETHER PROC. I	TOTAL γ		γ BY PROC. I	γ BY IRRAD. ETHER PROC. I	TOTAL γ	
1	30.4	1.0	31.4	75	60	16	76	59
2	11.5	5.0	16.5	50	24	18	42	51
3	7.7	7.0	14.7	75	18	20	38	31
4	28.0	1.0	29.0	150	92	12	104	50
5	11.0	2.0	13.0	75	41	13	44	41
6	3.4	2.0	5.4	38	14	4	18	33
							Average	44

*Extractions made immediately after addition of coproporphyrinogen to urine.

TABLE III. RECOVERY OF COPROPORPHYRINOGEN AFTER TREATMENT WITH IODINE*

AMOUNT I_2 ADDED IN γ	RECOVERY (γ)
40	36
80	62
120	91
160	92
200	91.6
400	60
500 (ether wash)	96.1
U.V. light 2′	29
U.V. light 4′	22

*Free coproporphyrin in a neutralized HCl solution was decolorized by shaking four minutes with sodium amalgam. After adding HCl to bring the PH to 7 to 8, the amounts of iodine shown in the table were added to aliquot samples, each of which contained 100 μg of chromogen. Three minutes later the samples were diluted with 0.3N HCl and the coproporphyrin was determined by fluorimetry.

efficient in this respect and that the porphyrin is relatively stable toward it. In Table III it is seen that the recovery of porphyrin starting from coproporphyrinogen is quite satisfactory when iodine is employed. The data in Table III are representative of the results of a number of titrations of pure coproporphyrinogen against iodine. These indicate that the reduction involves addition of six hydrogens to the porphyrin molecule, very probably including four to the CH bridges and two to nitrogens. At the same time, the data shown in Table III and IV reveal that there is an optimal range in the amount of iodine as well as the duration of oxidation for either coproporphyrinogen or native urinary chromogen. With large amounts of iodine added directly to the solution it is evident that some loss of porphyrin occurs. The most efficient method is seen to be the I_2 "wash," in which the ether or ethyl acetate extract is simply shaken once with 5 to 10 ml. of 0.005 per cent I_2, in 3 per cent sodium acetate, the iodine being extracted by the ethyl acetate and serving to oxidize the chromogen. The preformed porphyrin, as well as that

TABLE IV. THE RELATION OF I_2 CONCENTRATIONS AND TIME OF EXPOSURE TO EFFICIENCY OF CONVERSION OF CHROMOGEN TO PORPHYRIN

SAMPLE I		γ/24 HR.
1st run	Initial value	212
	Additional porph. by irrad. of ether	0
2nd run	+ 5 c.c. 0.02% I_2 for 5′*	240
3rd run	+ 5 c.c. 0.02% I_2 for 10′	250
4th run	+ 10 c.c. 0.02% I_2 for 5′	262
5th run	+ 10 c.c. 0.02% I_2 for 10′	270
6th run	+ 20 c.c. 0.02% I_2 for 5′	230
7th run	+ 20 c.c. 0.02% I_2 for 10′	210
8th run	+ 1 c.c. 1% I_2 for 1′	200
9th run	+ 2 c.c. 1% I_2 for 1′	180
10th run	+ 5 c.c. 1% I_2 for 1′	170
11th run	Ether washed once with 5 c.c. 0.02% I_2	276
SAMPLE II		
1st run	Initial value	300
	Additional porph. by irrad. of ether	20
	Total	320
2nd run	+ 10 c.c. 0.02% I_2 for 5′	350
3rd run	+ 20 c.c. 0.02% I_2 for 10′	320
4th run	+ 30 c.c. 0.02% I_2 for 10′	300
5th run	Ether washed once with 5 c.c. 0.02% I_2	367

*Iodine added to 100 ml. aliquots of urine, as indicated.

converted from chromogen, is then extracted by 1.5N HCl. Since the foregoing observations were made we have noted that Opsahl[16] used iodine to oxidize the chromogen of the uro-type porphyrins in porphyria urines. Whether this was porphobilinogen or another chromogen (see below) was not stated.

It is evident that difficulties which have been encountered with procedure I, i.e. unexplained increases or decreases from day to day on re-examination of the same sample or disagreement in results between different laboratories, are partially explained on the basis of occurrence of this chromogen. Other factors are discussed in the companion paper.[15] Two examples of the significance of this chromogen may be given briefly, as follows.

1. A freshly collected urine sample was subjected at once, in subdued light, to procedure II. Duplicate runs gave values of 5.0 and 5.1 γ per 100 milliliters. The urine was then allowed to stand for twenty-four hours, exposed to the ordinary light and air of the laboratory. Duplicate runs then gave values of 11.7 and 11.5 γ per 100 milliliters.

2. One hundred milliliters of a twenty-four hour sample from a case of hepatic cirrhosis was subjected to procedure I. This was done deliberately, over a five-hour period with frequent exposure to ultraviolet light in following the porphyrin during the extractions from urine to ether and ether to HCl. The value from this run was 531 γ per twenty-four hours. After ultraviolet radiation of the ether for two minutes, an additional 110 γ were found. Another 10 c.c. portion was then run in about thirty minutes, with minimal exposure to ultraviolet light. The value was 351 γ per twenty-four hours, with an additional 182 γ after two minutes' radiation of the ether. The ether was then washed once with 5 c.c. of 0.02 per cent I_2, after which more porphyrin was extracted corresponding to 53 γ for the twenty-four hour sample.

TABLE V. COMPARATIVE CONCENTRATIONS OF "PREFORMED" AND PRECURSOR FRACTIONS OF PORPHYRIN IN FRESHLY PASSED URINES

SAMPLE	PREFORMED (μG %)	PRECURSOR (μG %)	TOTAL (μG %)	PRECURSOR (%)
1	9.2	14.0	23.2	61
2	9.0	11.0	20.0	55
3	14.5	12.5	27.0	46
4	2.5	3.5	6.0	58
5	6.0	7.5	13.5	56
6	9.2	10.0	19.2	52
7	13.6	7.0	20.6	34
8	8.5	2.8	11.3	25
9	3.8	4.0	7.8	51
10	4.3	3.3	7.6	43
11	8.8	3.8	12.6	30
12	37.6	73.6	111.2	66
13	2.6	1.4	4.0	35
14	8.8	6.5	15.3	42
15	24.0	19.0	43.0	44
16	31.6	40.7	72.3	56
17	12.0	11.3	23.3	49
18	9.8	11.0	20.8	53
19	22.0	7.5	29.5	25
Mean				46

The proportion of porphyrin occurring as precursor in the freshly passed urine was determined in a series of individual urine samples having various concentrations of coproporphyrin. The preformed porphyrin was extracted from the ethyl acetate as used in procedure II, and was determined separately. The ethyl acetate was then washed once with 5 ml. of 0.005 per cent I_2 solution, after which porphyrin converted from chromogen was extracted and quantitated. The data are given in Table V. Since there can be little doubt that some conversion had already occurred during the initial mixing and ethyl acetate extraction, it is probable that, on the average, more than half of the total porphyrin is excreted as chromogen.

The effect of twenty-four hour refrigeration at −15° C. on preservation of the chromogen was studied in a series of nine individual urine samples, the same separation being employed as in obtaining the data for Table V. The results are given in Table VI. It is seen that there is a small but distinct reduction of precursor even under these conditions.

As shown in Table VII, on standing for twenty-four hours at room temperature little or no urinary chromogen remains. (The importance of maintaining an alkaline pH to prevent loss of porphyrin is considered in the companion paper.[15]) In these urines total porphyrin recovery was greater than that observed in the fresh samples, and the calculated average recovery of converted chromogen was 115 per cent. Because of this paradoxical figure a second series of urines was studied to compare the values for total coproporphyrin in freshly passed urine samples as determined by (1) separate extractions of preformed porphyrin and, following the "I_2 wash," of precursor converted to porphyrin, and (2) a single extraction of both preformed and precursor fractions following an "I_2 wash." These studies, as shown in Table VIII, yielded an average increase of 20 per cent with the "combined" method. This is believed to be due to a small loss of precursor during the extraction of the preformed porphyrin with HCl in the "separate" analysis, the large majority remaining in the ethyl acetate until after the I_2 wash. Similar studies of urines containing essentially no chromogen show no such difference between the totals obtained from separate or combined analyses. The presence

TABLE VI. THE EFFECT OF 24-HOUR REFRIGERATION ON URINARY COPROPORPHYRIN PRECURSOR

	PREFORMED PORPHYRIN (μG %)		PRECURSOR (μG %)		TOTAL (μG %)		PRECURSOR (μG %)	
SAMPLE	FRESH	FROZEN	FRESH	FROZEN	FRESH	FROZEN	FRESH	FROZEN
1	8.5	6.8	2.8	4.5	11.3	11.3	25	40
2	3.8	4.3	4.0	3.3	7.8	7.5	51	44
3	4.3	4.0	3.3	2.8	7.6	6.8	44	41
4	8.8	11.2	6.5	6.5	15.3	17.7	42	37
5	24.0	25.4	19.0	12.2	43.0	37.6	44	32
6	31.6	42.3	40.7	35.5	72.3	77.8	56	46
7	12.0	12.8	11.3	6.0	23.3	18.8	49	32
8	9.8	9.8	11.0	7.5	20.8	17.3	53	43
9	22.0	19.0	7.5	5.8	29.5	24.8	25	23
Mean							43	38

TABLE VII. A COMPARISON OF "PREFORMED" COPROPORPHYRIN AND CHROMOGEN IN FRESHLY PASSED URINE WHEN ANALYZED IMMEDIATELY AND AGAIN AFTER STANDING 24 HOURS AT ROOM TEMPERATURE

	FRESH URINE			AFTER STANDING 24 HOURS*			RECOVERY CONVERTED CHROMOGEN
SAMPLE	PREFORMED COPRO- (γ)	CHROMOGEN (γ)	TOTAL (γ)	PREFORMED COPRO- (γ)	CHROMOGEN (γ)	TOTAL (γ)	(%)
1	12.0	12.5	24.5	23.1	1.7	24.8	103
2	10.4	8.0	18.4	19.4	0.6	20.0	122
3	56.4	48.0	104.4	112.0	0.6	112.6	119
4	2.5	0.9	3.4	3.6	0.0	3.6	122
5	9.0	11.0	20.0	19.5	0.5	20.0	100
6	14.5	12.5	27.0	26.9	1.8	27.7	116
7	2.5	3.5	6.0	6.2	0.3	6.5	116
8	6.0	7.5	13.5	14.0	1.0	15.0	123

*Sodium carbonate added to fresh urine to achieve a concentration of 0.3 per cent.

of variable amounts of chromogen in the 1.5N HCl extract obtained from freshly passed urine can be demonstrated by re-extraction into ethyl acetate after neutralization of the HCl by sodium acetate. Additional amounts of porphyrin may then be obtained from this ethyl acetate by the use of iodine.

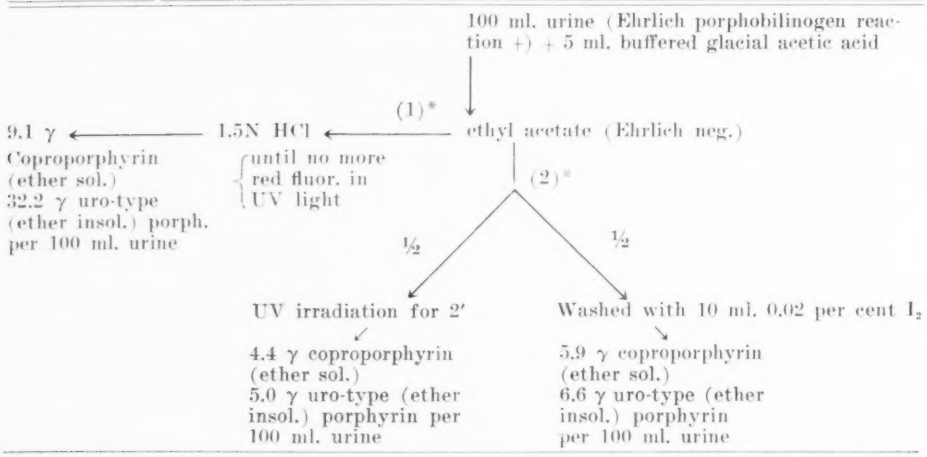
TABLE VIII. A COMPARISON OF TOTAL COPROPORPHYRIN RECOVERY FROM FRESHLY PASSED URINE WHEN PREFORMED COPROPORPHYRIN AND "PRECURSOR" ARE DETERMINED SEPARATELY OR TOGETHER

	SEPARATE ANALYSIS (μG/100 ML.)			COMBINED ANALYSIS (μG/100 ML.)	RATIO
SAMPLE	TOTAL (T_1)	PREFORMED	PRECURSOR	TOTAL (T_2)	T_2/T_1
1	23.2	9.2	14.0	27.8	1.20
2	12.6	8.8	3.8	20.8	1.65
3	111.2	37.6	73.6	128.0	1.15
4	4.0	2.6	1.4	3.7	0.93
5	27.0	14.5	12.5	29.5	1.09
6	6.0	2.5	3.5	7.0	1.17
7	13.5	6.0	7.5	16.5	1.22
Mean					1.20

A study of six urine samples from as many cases of the intermittent acute type of porphyria, and from one each of the photosensitive and mixed types, has demonstrated the presence of a chromogen or chromogens for uro-type porphyrins, distinct from porphobilinogen, the latter having been regarded hitherto as the sole precursor.[1] While porphobilinogen exhibits an Ehrlich reaction, the newly discovered chromogen is Ehrlich negative, in the concentrations thus far encountered, and is extracted from the urine at least in part by ethyl acetate, which does not extract porphobilinogen. This precursor is not appreciably removed from the ethyl acetate by 1.5N HCl, except after conversion to porphyrin by means of ultraviolet radiation.* The resultant porphyrin, however, may be distinguished readily by virtue of its insolubility

*We can fully confirm the recent observation of Gibson and Harrison[18] on the increase of uroporphyrin after heating the urine. A number of urine samples from the foregoing and additional cases of porphyria have shown from five to thirty fold increases after boiling for twenty minutes at pH 4.0. This treatment is much more efficient than either radiation or I_2 in converting precursor to porphyrin. Work by Miss Hawkinson, to be described elsewhere, clearly reveals that porphobilinogen (Ehrlich positive) and the precursor or chromogen of uroporphyrin (Ehrlich negative) may be separated quite completely, after which the former, on heating at pH 4.0, is converted only to porphobilin, the latter, however, to uroporphyrin.

TABLE IX. ETHYL ACETATE-SOLUBLE, EHRLICH NEGATIVE PORPHYRIN CHROMOGENS IN URINE OF CASE OF INTERMITTENT ACUTE PORPHYRIA (REMISSION)

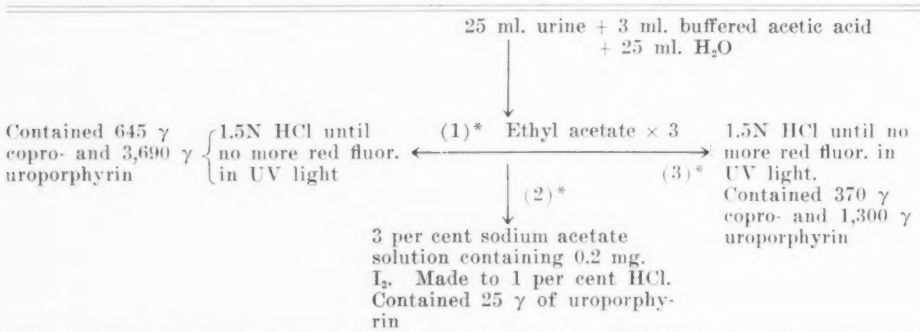

100 ml. urine (Ehrlich porphobilinogen reaction +) + 5 ml. buffered glacial acetic acid
(1)*
ethyl acetate (Ehrlich neg.)
1.5N HCl until no more red fluor. in UV light
9.1 γ Coproporphyrin (ether sol.) 32.2 γ uro-type (ether insol.) porph. per 100 ml. urine
(2)*
½
½
UV irradiation for 2′
Washed with 10 ml. 0.02 per cent I2
4.4 γ coproporphyrin (ether sol.) 5.0 γ uro-type (ether insol.) porphyrin per 100 ml. urine
5.9 γ coproporphyrin (ether sol.) 6.6 γ uro-type (ether insol.) porphyrin per 100 ml. urine

*Order of extractions.

in ether. Representative data are shown in Table IX. Verniory and co-workers[9] have recently stated in a brief communication that in a case of chronic porphyria associated with Hodgkin's disease, the urine exhibited a positive Ehrlich reaction, *no* uroporphyrin but increased amounts of coproporphyrin, which increased further on standing in the ether extract exposed to light and air. It was not entirely clear whether the chromogen in this instance was believed to be related to the Ehrlich reacting substances or was independent, as is evident in the present cases.

The urinary findings in the case of photosensitive (congenital) porphyria have been discussed in detail elsewhere.[17] The data relating to the chromogen are given in Table X.

TABLE X. ETHYL ACETATE-SOLUBLE PORPHYRIN CHROMOGENS FROM EHRLICH-NEGATIVE URINE OF CASE OF PHOTOSENSITIVE PORPHYRIA


25 ml. urine + 3 ml. buffered acetic acid + 25 ml. H2O
Contained 645 γ copro- and 3,690 γ uroporphyrin
1.5N HCl until no more red fluor. in UV light
(1)* Ethyl acetate × 3
(3)*
1.5N HCl until no more red fluor. in UV light. Contained 370 γ copro- and 1,300 γ uroporphyrin
(2)*
3 per cent sodium acetate solution containing 0.2 mg. I2. Made to 1 per cent HCl. Contained 25 γ of uroporphyrin

*Order of extractions.

In the present study a chromogen of coproporphyrin has been observed in the blood serum in three cases of porphyria, but it has not been possible to demonstrate it in serum or plasma of other individuals even though the urine

of some contained large amounts of both coproporphyrin and chromogen. In three cases of uremia in which the urine contained very small amounts of coproporphyrin (10.4, 11.4, and 3.0 γ per twenty-four hours) no chromogen was demonstrable in the urine, and neither porphyrin nor chromogen was demonstrated by suitable treatment of 20 ml. samples of serum. It is not clear whether this indicates destruction by some uremic metabolite, such as an oxyacid, or a sufficient continuous removal by the liver to prevent porphyrinemia.

TABLE XI. AMOUNTS OF COPROPORPHYRINOGEN IN A SERIES OF SAMPLES OF HUMAN FECES (FRESHLY PASSED)

DIAGNOSIS	PREFORMED COPROPORPHYRIN (γ PER 100 GM.)	COPROPORPHYRIN FROM CHROMOGEN* (γ PER 100 GM.)
1. Cirrhosis (with jaundice)	183	20
2. Cirrhosis (without jaundice)	60	10
3. Uremia	180	34
4. Myoglobinuria	196	8
5. Familial hemolytic jaundice	444	9
6. Uremia	288	21
7. Uremia	220	14
8. Hepatitis	59	59
9. Hemolytic anemia	300	30
10. Cirrhosis	320	15
11. Thrombocytopenic purpura	300	34
12. Hypochromic anemia	290	28

*Converted by I_2 wash.

As noted in Table XI, the present study indicates that as a rule but a small minority of the fecal coproporphyrin is excreted as a chromogen, but the possibility exists that considerable amounts of chromogen are converted to porphyrin during the extraction process, and thus go unrecognized. We have found it impossible to demonstrate a chromogen or precursor of coproporphyrin in fresh feces by means of ultraviolet radiation of ether or ethyl acetate extracts, after preliminary removal of preformed coproporphyrin with 1.5N HCl. It is probable, however, that the radiation in this instance catalyzes

TABLE XII. AMOUNTS OF COPROPORPHYRIN PRECURSOR IN HUMAN FISTULA BILE

SAMPLE	PERIOD OF COLLECTION	FIRST RUN		PERIOD OF STANDING BEFORE SECOND RUN	SECOND RUN	
		PREFORMED* COPROPORPH. (γ/100 C.C.)	CHROMOGEN* (γ/100 C.C.)		PREFORMED* COPROPORPH. (γ/100 C.C.)	CHROMOGEN* (γ/100 C.C.)
1	2 hr.	92	34	2 hr.	120	2
2	2 hr.	124	49	3 hr.	170	1.5
3	2 hr.	114	27			
4	2 hr.	97	36			
5	2 hr.	122	30			
6	12 hr.	95	3			
7	Immediate (by aspiration)	98	46	6 hr.	140	0
8	Immediate (by aspiration)	104	46	4 hr.	140	3.8
9	Immediate (by aspiration)	45	6	4 hr.	49	0.3

*Preformed and chromogen fractions determined by HCl extraction of primary ethyl acetate before and after "I_2 wash," as described in the text.

an oxidation beyond the porphyrin stage, as significant amounts of chromogen are regularly measurable when the afore-mentioned "I_2 wash" method is employed.

In one of Fischer's papers on phylloerythrin[19] it is stated briefly that this compound "in common with most of the other porphyrin of the feces, is excreted as a leukocompound." So far as can be determined, this is based upon the earlier finding of coproporphyrinogen in the feces of the case Petry, as referred to at the outset.

Studies of the chromogen in fistula bile samples, results of which are given in Table XII, have revealed wide fluctuations in amount, a larger proportion often being noted, however, than is true in the feces. It is also evident in Table XII that the chromogen is rapidly converted to the porphyrin as the bile stands after collection.

TABLE XIII. LACK OF RELATIONSHIP BETWEEN AMOUNTS OF CHROMOGEN AND ISOMER RATIO

SAMPLE	DIAGNOSIS	COPROPORPHYRIN (γ/DAY)		TYPE III PERCENTAGE (IN PREFORMED FRACTIONS)
		PREFORMED*	CHROMOGEN*	
1	Obst. biliary cirrhosis	357	50	6
2	Primary cholangiolitic cirrhosis	157	49	12
3	----------	139	21	48
4	Sporotrichosis and porphyria	477	209	54
5	Refractory (hyporegenerative anemia)	248	63	62
6	Hepatitis (?)	183	189	62
7	Porphyria	601	96	68
8	Lead poisoning	567	19	89

*Before and after I_2 wash of ethyl acetate extract.

The question arose as to whether the type I coproporphyrin differed from type III in respect to occurrence of chromogen. The data from various cases do not suggest this, as the variation in amount of chromogen was just as great with preponderance of one as with the other isomer. Representative data obtained from twenty-four hour collections of urine are given in Table XIII. Furthermore, determination of the isomer ratio on porphyrin obtained from chromogen by radiation has given essentially the same ratio as that found for the preformed porphyrin. (Table XIV.)

TABLE XIV. ISOMER FINDINGS IN PREFORMED COPROPORPHYRIN AS COMPARED WITH PORPHYRIN EXCRETED AS CHROMOGEN

SAMPLE	DIAGNOSIS	PERCENTAGE TYPE III ISOMER	
		IN PREFORMED FRACTION	IN CHROMOGEN
1	Hodgkin's disease	95	92
2	Porphyria	68	68
3	Cirrhosis	14	15

It will have been noted that throughout the foregoing discussion the terms chromogen and precursor are used interchangeably. The behavior of coproporphyrinogen as prepared in vitro by the amalgam reduction of the porphyrin, when compared with that of the nonfluorescing substance occurring

in the urine, both giving rise to porphyrin on treatment with iodine, suggests their identity but does not exclude the possibility of intermediate partially dehydrogenated compounds. In support of the latter possibility is the chromatographic observation on two occasions of colored, nonfluorescent zones which developed red fluorescence on exposure to ultraviolet light. Furthermore, the addition of coproporphyrinogen to six urines gave an average recovery of only 77 per cent when employing procedure II plus the iodine wash, whereas, as described earlier, complete recovery of the naturally occurring urinary chromogen is obtained by this method. Further studies of the properties of the urinary chromogen are in progress.

SUMMARY AND CONCLUSIONS

The coproporphyrin of human urine is excreted largely as a chromogen or precursor, which is similar in many ways to that which is readily prepared, in vitro, by the amalgam reduction of crystalline coproporphyrin. It is probable that this finding simply confirms the long-neglected observation of Saillet, though his data do not permit accurate comparisons.

The chromogen is readily extracted from urine by ether or ethyl acetate but not more than small fractions are extracted from these solvents by 1.5N hydrochloric acid. Since it does not fluoresce, it is readily overlooked and discarded unless methods are employed to convert it to the porphyrin and extract it as such.

The chromogen is converted to the porphyrin rather inefficiently by brief ultraviolet radiation. Continued radiation causes progressive deterioration. Iodine, in a ratio of 3 molecules of I_2 to 1 of coproporphyrinogen, converts this chromogen almost quantitatively to the porphyrin.

In relatively fresh urine, porphyrin determinations carried out rapidly, without use of iodine and with but little exposure to light, give significantly lower values than those on the same samples carried out deliberately and with considerable light exposure. Differences of this type are believed to explain certain inconsistencies encountered with the quantitative methods heretofore employed, none of which have taken the chromogen into account.

The amount of chromogen has varied without relation to the isomer preponderance (whether I or III); isomer analyses on porphyrin excreted as chromogen have revealed the same ratio in any given urine sample as that found for the preformed porphyrin.

The chromogen is demonstrable in but small proportion in the feces, but this may be due to conversion during extraction. Larger proportions of chromogen are observed in fresh fistula bile; here it is evident that conversion to the porphyrin occurs rapidly as the bile stands after collection.

ADDENDUM

Since completion and preliminary communication[20] of the foregoing work, an abstract of a study of the urinary chromogen of coproporphyrin has been published by Raine.[21] In this the use of quinhydrone is recommended for conversion of chromogen to porphyrin. In our hands this is not as efficient as the small amounts of iodine presently described. Gray[22] has more recently suggested that the precursor may be a metal com-

plex. This suggestion apparently was based on the greater concentration of HCl required to extract metal complexes from an ethereal solation. At the outset of our investigations of possible causes of the discrepancy in values obtained with the afore-mentioned procedures I and II, we carefully considered this possibility but rejected it on the following grounds. (1) The zinc complex of coproporphyrin, which is often encountered in various urine samples, is readily split by 1.5*N* HCl, which, however, extracts but small amounts of the chromogen; the zinc complex, furthermore, exhibits fluorescence, unlike the chromogen. (2) The copper complex, which is not fluorescent and not extracted from ether by HCl, could not be found in suitable Na OH concentrates from the ethereal extracts of large amounts of precursor containing urine, nor was any metal complex spectrum observed. (3) Finally, the efficiency of iodine in conversion is hardly compatible with the behavior of the known metal complexes.

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AN IMPROVED METHOD FOR THE DETERMINATION OF URINARY COPROPORPHYRIN AND AN EVALUATION OF FACTORS INFLUENCING THE ANALYSIS

SAMUEL SCHWARTZ, M.D., LESLIE ZIEVE, M.D., AND CECIL JAMES WATSON, M.D.
MINNEAPOLIS, MINN.

DURING the past half century there has accumulated a sizeable literature dealing with the determination of urinary coproporphyrin. Most of the quantitative methods employed have been modifications of that described by Fikentscher,[1] involving repeated extraction of 50 to 100 ml. of urine by ether, varying degrees of further purification, and fluorimetric analysis. A critical evaluation of these methods, however, has revealed that all have a number of serious deficiencies in varying degree. (1) They are time consuming. (2) They do not take into account the excretion of a large part of the urinary coproporphyrin as a nonfluorescent precursor. (3) Emulsions formed on extracting large volumes of urine with ether are troublesome. (4) Optimum conditions for the preservation of urinary porphyrin have not been defined. (5) Factors of importance in the fluorimetric analysis have not been considered adequately.

It is the purpose of the present communication to consider these various factors and to describe a simplified procedure for the determination of urinary coproporphyrin. This method now has been used in our laboratories for about two years. It appears to have eliminated these deficiencies, has yielded more consistently accurate results, and has shortened the time of analysis by a factor of approximately three.

The general nature and significance of the precursor or chromogen of urinary coproporphyrin are discussed in the companion paper.[2] Only those aspects of this subject which relate directly to the new method to be described will be considered here.

ANALYTICAL METHODS

1. *Improved Procedure.—*

(a) *Collection of Urine:*

Five grams of sodium carbonate are added to the collecting bottle to insure an approximately neutral or alkaline pH in those instances where a twenty-four hour collection of urine is to be made. The bottle may be either colorless or amber. An amber bottle is recommended in those instances where urobilinogen is to be determined in the same speci-

From the Department of Medicine, University of Minnesota and Veterans Administration Hospital.

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men. If single samples are to be analyzed, sufficient sodium carbonate should be added to achieve a concentration of 0.3 to 1.0 per cent. The urines need not be refrigerated unless the analysis is delayed more than several days.

(b) *Extractions:*

To 5 ml. of the well-mixed urine in a separatory funnel, add 5 ml. of buffered acetic acid solution,* 75 to 100 ml. of ethyl acetate, and 15 to 20 ml. of distilled water. Shake well. Discard the aqueous phase. Wash the ethyl acetate twice with 20 to 30 ml. portions of 1 per cent sodium acetate and once with 0.005 per cent aqueous iodine.† The porphyrin is then extracted from the ethyl acetate with four portions of about 5 ml. each of 1.5N hydrochloric acid. If the fourth hydrochloric acid extract still has any reddish-orange fluorescence when irradiated in the dark with near-ultraviolet light, the hydrochloric acid extractions are continued until they show no fluorescence. Maximum fluorescence is excited by the 405 mμ light from a mercury arc lamp.‡ The combined hydrochloric acid extracts are diluted with 1.5N hydrochloric acid to 25 ml. or some other convenient volume, and the fluorescence intensity is measured in a sensitive fluorimeter.

With many hundreds of different urine samples, no emulsions have been encountered on shaking the acidified urine with ethyl acetate except in a single instance where a considerable amount of albumin was present. A small amount of precipitate which occasionally collects at the interphase is removed by the 1 per cent sodium acetate washes.

The 1.5 N hydrochloric acid extracts are essentially colorless except in some instances where considerable bile pigment is present in the urine. Even in these cases the intensity of color is less than that obtained with the previously used "long" method. Prior to fluorimetry, colored extracts are further diluted with 1.5N hydrochloric acid until nearly colorless when viewed through the side of the tube.

If a slight opalescence is seen in the hydrochloric acid extract, it may be removed by further dilution with 1.5N hydrochloric acid.

If uro-type porphyrins are present in the urine, as in cases of porphyria, a variable fraction is extracted by the ethyl acetate. This is largely removed by the sodium acetate washes. The presence of red fluorescence in these washes is clearly indicative of a uro-type porphyrin. This finding, or the presence of a positive porphobilinogen reaction in the native urine, will indicate the need of special investigation with respect to abnormal uro-type porphyrins.

If the fluorimeter available is insufficiently sensitive, i.e. if it is impossible to set a standard containing 10 μg of coproporphyrin per 100 ml. to read 100, one may use a smaller final volume of hydrochloric acid solution. It is also permissible to use up to approximately 10 ml. of urine initially, with 125 ml. of ethyl acetate for the extraction.

In order to ascertain the importance of various technical factors, analyses have been made by a number of procedures which were slight modifications of that described here. These will be reported below to the extent that they illustrate phenomena of importance to the interpretation of the method or of previously reported data from this and other laboratories.

(c) *Fluorimetry:*

(1) A modified Farrand fluorimeter:

Because commercially available fluorimeters were generally too insensitive for accurate work with 5 ml. of urine,§ a modified apparatus employing a photomultiplier tube was constructed as follows.

Using a Klett mercury light source, lamp housing, and condensing lens, a light-tight wooden box was constructed to house the primary and secondary filters and an Evelyn colorimeter tube as shown in Fig. 1. The box and test tube holder were coated with dull black paint to limit reflection of light. A IP21 photomultiplier tube with battery power

*Four volumes of glacial acetic acid plus one volume of saturated aqueous sodium acetate solution.

†Prepared by daily dilution with water of a refrigerated stock 1 per cent solution of iodine in alcohol.

‡Model DL-2, Black Light Products, Chicago, Ill.

§Calectron, Inc., 2444 Beverly Road, St. Paul 4, Minn., has agreed to manufacture a moderately priced fluorimeter meeting our specifications for coproporphyrin determination.

supply* was fixed to the exit slit. Readings were made with a moderately sensitive galvanometer (1 mm. deflection per 0.005 microamperes) by setting the blank at zero and the standard at 100. Provision was also made for the use of a second light source, shown to the left in Fig. 1, where other exciting wave lengths may be desired. The appropriate light source is chosen by rotating the test tube adapter.

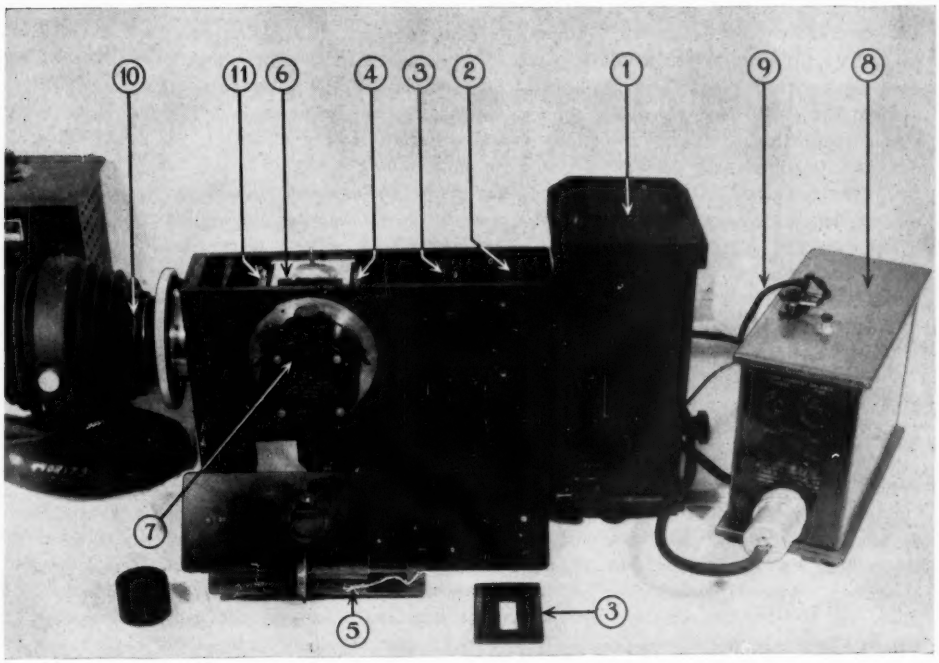

Fig. 1.—A modified Farrand fluorimeter. *1*, Mercury lamp and housing; *2*, condensing lens; *3*, light shields with slits to transmit parallel light when using interference filters; *4*, primary filter; *5*, test tube holder; *6*, secondary filter; *7*, photomultiplier tube; *8*, battery power supply for photomultiplier tube; *9*, leads to galvanometer; *10* and *11*, another light source and another set of primary filters may be employed for fluorescence measurements where the mercury lamp is deficient in the proper excitation wave lengths.

To determine the porphyrin concentration of test samples, the coproporphyrin standard solution is set to read 100 with a blank reading of zero, the fluorescence intensity of the unknown is measured, and its concentration is determined by reference to a calibration curve prepared from solutions of known coproporphyrin concentration. Both unknown and standard solutions should be at room temperature.

While the sensitivity of different IP21 photomultiplier tubes varies, the three tested in our laboratory have given a 100 mm. deflection on the galvanometer with as little as 1 to 4 μg of coproporphyrin per 100 ml. of solution.

(2) A modified Beckman fluorimeter:

As an alternative to the foregoing apparatus, the light source and box may be attached to a Beckman spectrophotometer, model DU,† so that the exit slit from the box is adjacent to the entrance slit of the phototube compartment. A 10,000 megohm resistor† is substituted in the latter for the usual 2,000 megohm resistor to give sensitivity similar to that obtained with the modified Farrand fluorimeter.

To determine fluorescence intensity, the 1.5N hydrochloric acid blank in an Evelyn tube is placed in the adapter, transmission is set at zero, and the needle is centered by

*Farrand Optical Company, New York, N. Y.
†National Technical Laboratories, South Pasadena, Calif.

means of the dark current adjustment. The porphyrin standard is next set to give a transmission reading of 100 by adjusting the sensitivity knob. The transmission reading of the unknown is then determined. It will be noted that no use is made here of the light source or monochromator of the Beckman apparatus.

(3) Filters:

It is important that the secondary filter absorb essentially all the light transmitted by the primary filter to avoid errors due to reflection of light from the walls of the test tube or test tube holder. To achieve this goal, most of the studies described herein employed as primary filters a 405 mμ interference filter* and a blue auxiliary filter which absorbs most of the 589 mμ light also transmitted by the interference filter. The secondary red filter used (Corning #2-61) has negligible transmission below 600 mμ. With this combination of filters, the blank solution before setting at zero gives about 0.3 mm. deflection with a 10 μg per cent solution set to read 100.

Greater sensitivity and equal accuracy with the improved method is obtained by the use of Corning filters #5-58 or 5-60 to transmit the 405 mμ exciting light. Corning filters #2-61 or 2-62 may then be used as secondary filters. These filter combinations give a blank deflection of 5 to 7 mm. before setting at zero. The transmission properties of these filters as well as the relation of primary filters to the form of the calibration curve and to the accuracy of analysis are discussed in Experimental sections 3, 6b, and 8b below.

(4) Coproporphyrin standard:

As noted in the experimental section below, the presence of ethyl acetate dissolved in the 1.5N hydrochloric acid extracts enhances the porphyrin fluorescence by about 8 per cent. To correct for this factor most simply, it is recommended that the coproporphyrin standard be 8 per cent more concentrated than designated. Thus, for example, a 10.0 μg per 100 ml. standard would actually contain 10.8 μg per 100 milliliters.

Previous communications[3, 4] have reported the use of fluorescein standards. Such standards are no longer recommended since both excitation and fluorescence spectra of fluorescein differ markedly from those of coproporphyrin. Every change in filters employed therefore requires recalibration of the fluorescein standard against reference solutions of coproporphyrin. The use of coproporphyrin standards obviates this potential source of error. The coproporphyrin standards in corked, paraffin-sealed tubes have been found to be quite stable even when kept at room temperature and used several times daily. They are checked at weekly or monthly intervals against similar solutions stored in a refrigerator.

2. *Previously Used "Long" Method.*—

The previously used modification of Fikentscher's method, to be called here the "long" method, has been described in detail elsewhere.[3] Briefly, it involves acidification of 100 ml. of urine with glacial acetic acid, and extraction of the porphyrin with three portions of ether. The combined ethers are washed three times with 3 per cent sodium acetate and the porphyrin is extracted exhaustively into 5 per cent hydrochloric acid (prepared in the proportion of 5 ml. of concentrated or 37 per cent hydrochloric acid diluted to 37 ml. with distilled water). The hydrochloric acid solution is neutralized with sodium acetate and the porphyrin re-extracted with three portions of ether. Following three water washes, the porphyrin is removed by repeated extractions with 1 per cent hydrochloric acid and its concentration is determined fluorimetrically.

EXPERIMENTAL

1. *Recovery Experiments.*—

(a) *Recovery of Pure Coproporphyrin From Dilute Hydrochloric Acid:*

In a series of experiments, 0.1 to 3.0 μg of pure coproporphyrin in 0.3N hydrochloric acid were diluted to 5 ml. with 3 per cent sodium acetate to neutralize the hydrochloric acid, then extracted by the above-described procedure. These amounts correspond to those found in 5 ml. of urine where the urinary

*Baird Associates, Cambridge, Mass.

coproporphyrin concentration ranges from 2 to 60 μg per 100 milliliters. As shown in Table I, recoveries ranged from 103 to 112 per cent, with an average of 107 per cent.

Such a large recovery seemed paradoxical at first. It was found, however, that the presence of ethyl acetate or ethyl ether in the hydrochloric acid solution of the coproporphyrin augments its fluorescence by about 8 per cent. This was determined by adding 1.0 to 1.5 ml. of either ethyl acetate, ether, or 1.5N hydrochloric acid to triplicate 20 ml. portions of hydrochloric acid solutions of coproporphyrin standard. These solutions are completely miscible. In every instance the ethyl acetate- or ether-treated sample read from 6 to 10 per cent more than the sample to which only the same volume of 1.5N hydrochloric acid had been added. Since the coproporphyrin standards used to calculate the amount of initial porphyrin in the recovery study contained only dilute hydrochloric acid whereas the recovered samples were essentially saturated with ethyl acetate, one would expect the latter to have approximately 8 per cent more fluorescence, assuming quantitative recovery.

TABLE I. RECOVERY OF PURE COPROPORPHYRIN FROM DILUTE HYDROCHLORIC ACID

SAMPLE	COPROPORPHYRIN ADDED (μG)	COPROPORPHYRIN RECOVERED (μG)	PER CENT RECOVERED
1	0.10	0.11	110
2	0.30	0.33	110
3	0.36	0.38	105
4	0.36	0.37	103
5	0.60	0.62	103
6	1.00	1.12	112
7	1.50	1.68	112
8	2.00	2.16	108
9	2.50	2.60	104
10	3.00	3.20	107
		Av.	107

Confirmatory evidence for this observation was obtained in a second series of recovery determinations in which the coproporphyrin had been previously extracted from ether by hydrochloric acid to enhance its fluorescence. The concentration of porphyrin in this hydrochloric acid solution was determined and used as a basis for comparison in eight samples taken for re-extraction and recovery analysis. No further enhancement of fluorescence would be expected under these conditions. Six of the recoveries now ranged from 97 to 101 per cent. The other recoveries were 85 and 105 per cent. The average was 99 per cent.

(b) *Recovery of Pure Coproporphyrin Added to Urine:*

Various amounts of coproporphyrin were added to normal urines and to urines containing large amounts of bile pigment or of albumin. The results are summarized in Table II in terms of the average value for triplicate analyses. As shown in Table II, an average recovery of 108 per cent of the added porphyrin was obtained, without correction for the fluorescence enhancement by the ethyl acetate.

TABLE II. RECOVERY OF COPROPORPHYRIN ADDED TO URINE
(AVERAGES OF TRIPLICATE ANALYSES)

NO.	URINE COPROPORPHYRIN (μG PER 5 ML.)	COPROPORPHYRIN ADDED (μG)	TOTAL RECOVERED (μG)	PER CENT ADDED COPROPORPHYRIN RECOVERED
1	0.58	0.72	1.39	113
2	1.32	1.68	3.15	109
3	1.32	0.84	2.19	104
4	3.48	2.40	6.25	115
5	3.48	4.80	8.90	113
6	0.33	0.36	0.76	119
7	0.33	0.71	1.09	107
8	1.14	1.13	2.28	101
9	1.14	2.26	3.28	95
			Av.	108

2. *Reproducibility of Results.*—

Triplicate analyses were performed on sixteen urines with or without the addition of coproporphyrin. In these samples the mean values for the triplicate analyses ranged from 1.6 to 178.0 μg per 100 milliliters. The three individual analyses of each specimen differed from their mean by an average of 2.9 per cent. The range of their mean differences was 0.3 to 7.3 per cent. There was no significant correlation between the percentage variability in the analyses and the porphyrin concentration.

Duplicate analyses were carried out on thirty-three urines by four different technicians. The range of differences in the duplicates was zero to 8.7 per cent, with an average of 3.4 per cent.

3. *Spectral Purity of Fluorescence in the 1.5N Hydrochloric Acid Extracts.*—

Because of the low concentration of porphyrin in the 1.5N hydrochloric acid extracts, we have not yet been able to study the fluorescence spectra of the samples directly. Indirect evidence on this point was obtained by employing three different primary filters* for each analysis. For each primary filter three different secondary filters* were used. The same coproporphyrin standard was used in each instance. The transmission properties of these filters are summarized in Table III.

TABLE III. TRANSMISSION PROPERTIES OF PRIMARY AND SECONDARY FILTERS EMPLOYED

FILTER	PER CENT TRANSMISSION AT 360 mμ	400 mμ	600 mμ	620 mμ	640 mμ
I° 5-60 (5543)	11	60	0	0	0
5-58 (5113)	3	38	0	0	0
7-54 (9863)	84	12	0	0	0
II° 2-61 (2412)	0	0	1	52	84
2-62 (2418)	0	0	19	77	88
2-63 (2424)	0	0	57	85	88

As shown in Table IV, the 1.5N hydrochloric acid extracts gave essentially the same galvanometer readings with primary filters #5-60 and 5-58 regardless of the secondary filter used. Only slight differences were observed with the least specific primary filter, #7-54. If other interfering fluorescing com-

*Corning Glass Works, Corning, N. Y.

TABLE IV. FLUORESCENCE INTENSITIES OF NINE URINARY COPROPORPHYRIN EXTRACTS (NEW METHOD) MEASURED WITH VARIOUS FILTER COMBINATIONS

CORNING FILTER		GALVANOMETER READINGS								
I°	II°	1	2	3	4	5	6	7	8	9
5-60	2-61	75.5	36.3	10.0	28.0	47.8	44.3	21.5	14.8	27.3
5-60	2-62	75.0	36.5	10.3	28.3	48.0	44.5	21.8	15.0	27.8
5-60	2-63	75.5	36.5	10.5	28.3	47.8	44.5	22.0	15.0	27.8
5-58	2-61	75.3	36.3	10.0	27.8	47.5	45.0	21.3	15.3	27.3
5-58	2-62	75.3	36.8	10.5	28.0	47.5	45.3	21.3	15.5	27.5
5-58	2-63	75.3	36.8	10.5	28.3	47.3	44.5	21.8	15.3	27.5
7-54	2-61	74.5	34.0	12.5	29.0	45.0	45.0	22.0	15.0	27.0
7-54	2-62	73.5	34.5	12.5	29.5	46.0	46.0	22.0	15.5	27.5
7-54	2-63	73.5	34.0	13.0	30.3	46.0	45.0	22.8	15.0	27.0

Values listed are galvanometer readings to the nearest ¼ mm., rounded off to tenths.

pounds had been present in appreciable amount, one would expect that their excitation or fluorescence spectra would differ sufficiently from that of the coproporphyrin standard to yield different readings with the different filter combinations employed.

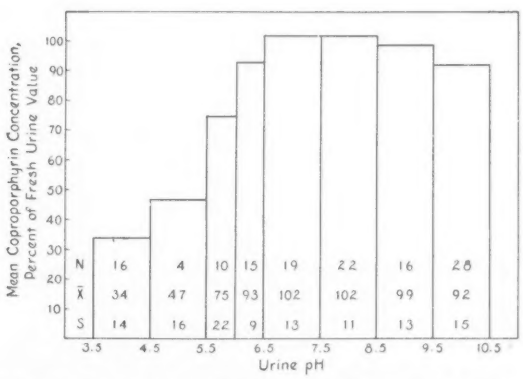

Fig. 2.—The effect of pH on the preservation of coproporphyrin in urine kept at room temperature for twenty-four hours. *N*, number of urines analyzed; $\bar{X}$, mean; *S*, standard deviation.

4. *The Effect of pH on Recovery of Urinary Coproporphyrin.*—

The concentration of coproporphyrin was determined in thirty-four freshly passed urines. Sodium hydroxide or hydrochloric acid was then added to alter the pH of aliquot samples within a range of 3.5 to 10.5. Varying amounts of sodium carbonate, sodium bicarbonate, and sodium phosphate were added to other aliquots of the same urines to attain an approximately neutral or slightly alkaline pH. In each instance the pH was determined with a pH meter immediately after addition of acid or alkali, and again after the urines had stood at room temperature for twenty-four hours. Only slight increases in pH occurred during this interval. The porphyrin analyses were then repeated. Results at consecutive pH ranges are summarized in Fig. 2 in terms of the recovery of urinary coproporphyrin, related to the value for the fresh sample. As shown in Fig. 2, urines kept twenty-four hours at a pH of 6.5 to 9.5 show no significant change in urinary porphyrin concentration. With increasing acidity, marked losses of porphyrin occur, while small losses are observed at pH's over 9.5.

Sodium carbonate was found to be the most convenient alkalinizing agent. Its effect on the preservation of urinary porphyrin was studied further in a series of forty-seven freshly passed urines which were divided into two equal portions. Sufficient sodium carbonate to yield a concentration of 0.3 to 1.0 per cent was added to one half, and nothing to the other. The coproporphyrin concentration was determined at the end of twenty-four hours by the improved method in all forty-seven, and by the "long" method in nineteen of these. Results by the two methods were in excellent agreement; the carbonate-containing fractions showed, on the average, a 65 per cent greater coproporphyrin concentration than the non-carbonate-treated samples (or, conversely, the latter showed a 39 per cent loss). There was marked variation about this mean (standard deviation = 74 per cent) which was obviously due to the wide variation in the initial pH, the more acid urines exhibiting much greater loss when carbonate was not used.

Maechling[5] suggested that part of the urinary porphyrin is excreted as a conjugated compound which is hydrolyzed at room temperature by one-tenth volume of concentrated hydrochloric acid, but not by 10 per cent acetic acid. She reported that when urine was acidified for two hours with hydrochloric acid, larger amounts of coproporphyrin were found than when acetic acid was used. No comparison was made with urine to which no acid was added. It was found in the present study that hydrochloric acid resulted in a mean loss of 39 per cent within two hours (six analyses, range 14 to 73 per cent). In agreement with Maechling's data, losses in aliquot portions acidified with glacial acetic acid were even larger.

5. *Stability of Urinary Coproporphyrin in the Presence of Sodium Carbonate.*—

In a series of eleven urines preserved with 0.3 per cent sodium carbonate, total coproporphyrin concentration was determined at twenty-four, forty-eight, and ninety-six hours. No significant differences were found. The average ratios of the values at these intervals to those obtained for the fresh urine were, respectively, 1.0 ± 0.09, 0.98 ± 0.10, and 1.06 ± 0.19, where the numbers indicating variation about the means here and below are standard deviations. One aliquot of each of these urines was frozen at once and another after standing twenty-four hours. Two weeks later they were thawed and analyzed. The corresponding ratios were 1.0 ± 0.15 and 1.06 ± 0.11, respectively. It is evident that in an appropriately neutral or alkaline urine, coproporphyrin is quite stable.

6. *Factors Influencing Fluorescence Intensity.*—

(a) *Porphyrin Concentration:*

At concentration levels of less than 5 μg of coproporphyrin per 100 ml., one finds a linear relationship between porphyrin concentration and galvanometer reading. At higher concentrations there is increasing departure from linearity until, with the apparatus and filter combinations recommended, maximum readings are obtained with concentrations of approximately 140 μg per cent. As shown in Fig. 3, further increases in concentration result in

marked fluorescence-quenching and decreased galvanometer readings, due chiefly to light absorption by the porphyrin. As one views the fluorescing solution from above, one notes that fluorescence is excited in the latter instances only at the edge of the tube facing the light source.

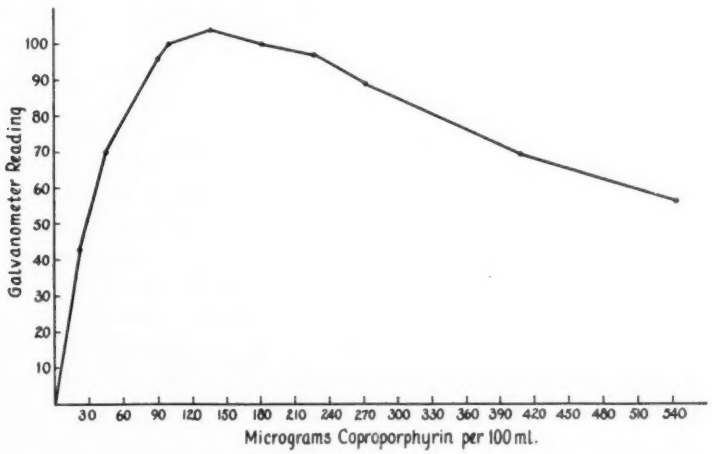

Fig. 3.—The relation of coproporphyrin concentration to fluorescence intensity. All readings made with reference to a standard 100 μg per cent solution of coproporphyrin set to read 100.

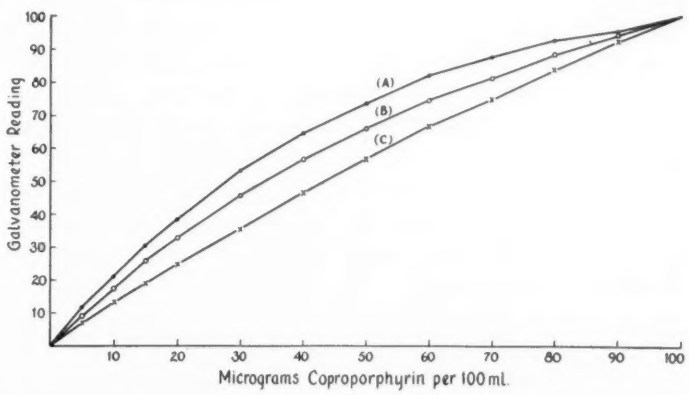

Fig. 4.—The relation of coproporphyrin fluorescence to exciting wave lengths of ultraviolet light. (*A*), 405 mμ interference filter and auxiliary blue filter; (*B*), Corning filter #5-57 (transmits 70 per cent at 365 mμ and 84 per cent at 405 mμ); (*C*), 365 mμ interference filter.

It is apparent from Fig. 3 that the use of a standard containing 100 μg of coproporphyrin per 100 ml. is subject to a potentially large error in the analysis of high concentrations of porphyrin. This is especially true in the presence of colored impurities which will add to the quenching of fluorescence by the porphyrin. In such samples, a galvanometer reading of 90 to 100 could indicate a concentration of approximately 70 to 250 μg per cent. This error is avoided by diluting the solution until it is essentially colorless and by using a less concentrated standard.

(b) *Wave Length of Exciting Light:*

To illustrate the importance of the exciting wave length on the shape of the fluorescence calibration curve, a 100 μg per cent standard solution of coproporphyrin in 0.3N hydrochloric acid was set to read 100, and readings were taken at various other concentrations employing different primary filters. As shown in Fig. 4, fluorescence intensities varied depending upon the wave length of the exciting light transmitted by the different filters.

When the same filters were employed, identical curves were obtained using either a Lumetron fluorimeter or the IP21 phototube apparatus described herein. The combined use of filters *A* and *B* of Fig. 4 also yielded the same curve as that obtained by the use of *A* alone, since in both instances only the 405 mμ light was transmitted; its intensity was merely reduced.

The importance of a proper choice of exciting light in quantitative urinary porphyrin analyses is discussed in Section 8 below. For those interested in qualitative or semiquantitative analyses of porphyrin, it should be pointed out here that, in impure solution, irradiation with diffuse near-ultraviolet light may yield shades of blue, green, bluish-white, or violet fluorescence even in the presence of considerable amounts of porphyrin. Excitation with 405 mμ light alone, however, will yield purer orange or red fluorescence since the fluorescence of most interfering substances is excited chiefly by the 365 mμ light.

(c) *Hydrochloric Acid Concentration:*

Porphyrin fluorescence diminishes with increasing hydrochloric acid concentration. In addition, the relative amount of fluorescence which is quenched by a given concentration of hydrochloric acid varies with the porphyrin concentration, as shown in Table V. The data in Table V were prepared by comparing the fluorescence of equal concentrations of coproporphyrin in 0.3N and 1.5N hydrochloric acid. In each instance the fluorescence of the 1.5N solution was determined with the corresponding 0.3N solution set to read 100. In this series the ratio of fluorescence intensity, comparing the 0.3N to the 1.5N hydrochloric acid solution, ranged from 1.10 to 1.26 at porphyrin concentrations of 5 to 100 μg per 100 milliliters.

TABLE V. THE EFFECT OF PORPHYRIN CONCENTRATION ON THE RELATIVE INTENSITY OF FLUORESCENCE IN 0.3N AND 1.5N HYDROCHLORIC ACID

PORPHYRIN CONCENTRATION (μG %)	GALVANOMETER READING		RATIO 0.3N/1.5N HCl
	0.3N HCl*	1.5N HCl	
5.0	100	90.5	1.10
12.5	100	90.3	1.11
25.0	100	88.8	1.13
50.0	100	86.0	1.16
75.0	100	82.5	1.21
100.0	100	79.3	1.26

*Solutions of porphyrin in 0.3N HCl arbitrarily set to read 100 in each instance.

(d) *Saturation of Final Extract With Ethyl Acetate:*

As indicated earlier, saturation of the hydrochloric acid extract with ethyl acetate enhances fluorescence by approximately 8 per cent. This phenomenon

has been observed with other organic solvents such as ethyl ether and butyl alcohol.

(e) *Temperature:*

At porphyrin concentrations of 2 to 850 μg per 100 ml. of 0.3N hydrochloric acid, a diminution in fluorescence intensity of 4 to 5 per cent was found for each 10° C. elevation of temperature in the range from 10° to 50° C. This is presumably due to greater loss of excitation energy by molecular collisions at elevated temperatures.

7. *Reasons for Choice of Reagents, Volumes, and Concentrations Employed in the Improved Method.—*

(a) *Sodium Carbonate Preservative:*

As indicated earlier, sodium carbonate was found to be a satisfactory preservative. Optimum carbonate concentrations in urine ranged from 0.3 to 1.0 per cent, though concentrations as high as 5.0 per cent have been found satisfactory. The effect of any given concentration is influenced by the initial pH and buffering capacity of the urine. However, maximal recoveries of coproporphyrin are obtained over a relatively wide pH range as indicated in Fig. 2. Five grams of sodium carbonate yields, generally, a final concentration of approximately 0.3 to 0.5 per cent in the twenty-four hour urine sample. The concentration in the first voided specimen rarely exceeds 5.0 per cent. Occasionally appreciable loss of coproporphyrin results from the use of this amount of sodium carbonate. The ideal preservative remains a subject for further study.

(b) *Volume of Urine:*

A small volume of urine is desirable because there is less tendency for emulsion formation and less interference from impurities which may mask or quench porphyrin fluorescence. The limiting factor is the sensitivity of the fluorimetric analysis. Though 5 ml. of urine is to be preferred, 10 ml. may be used, as indicated by the following studies. Twelve urines were analyzed in duplicate using both 5 ml. and 10 ml. of urine. Seventy-five milliliters of ethyl acetate were used in the former instance and 125 ml. in the latter. An insignificant mean difference of 3 per cent (range 0 to 16 per cent) was obtained in favor of the 10 ml. volumes. In six additional samples, a comparison was made of 5 ml. and 20 ml. of urine. A significant mean loss of 14 per cent (range 5 to 20 per cent) was obtained with the 20 ml. samples.

(c) *Buffered Acetic Acid:*

Water will extract appreciable amounts of coproporphyrin from ether containing large amounts of glacial acetic acid. Though the porphyrin is more soluble in ethyl acetate, even here some loss occurs at high acetic acid concentration. The addition of one-fourth volume of saturated aqueous sodium acetate to glacial acetic acid yields a Congo red reacting solution which insures against porphyrin loss in the aqueous phase, with the exception of uroporphyrin.

(d) *Ethyl Acetate:*

As noted, the urinary porphyrin is more soluble in ethyl acetate than in ether. In addition, ethyl acetate is nonexplosive and does not form peroxides.

A relatively larger volume of ethyl acetate is used to insure complete extraction of the porphyrin. Its one disadvantage is that of greater expense, but it may be recovered by washing with 10 per cent sodium carbonate, dehydration overnight with calcium chloride, and distillation over fresh calcium chloride. Comparative analyses using this distilled ethyl acetate and new ethyl acetate have been satisfactory.

(e) *One Per Cent Sodium Acetate Wash:*

Duplicate analyses were performed on thirty-two urine samples, employing distilled water washes of the ethyl acetate in one and 1 per cent sodium acetate washes in the other. An average difference of 4 per cent was found between the duplicates. The mean value obtained with the water washes was 230 μg per day, and with the sodium acetate washes, 231 μg per day. The mean absolute difference was 1.1 $\pm$ 14.8 μg per day. Nonetheless, 1 per cent sodium acetate washes are recommended since they remove more green-fluorescing material and result in purer orange-fluorescing hydrochloric acid extracts when viewed in diffuse near-ultraviolet light, such as generally employed qualitatively. This is especially true when working with icteric urines. When excited with only 405 mμ light, as in the quantitative method, the color of the fluorescence is about the same. Excellent checks were also obtained by the use of 3 per cent sodium acetate washes.

(f) *0.005 Per Cent Iodine:*

The importance of the iodine wash for conversion of the nonfluorescent coproporphyrin precursor is discussed in the companion paper.[2] Though this precursor, or chromogen, accounts for roughly half the total porphyrin in the freshly passed urine, it is nearly all converted to porphyrin within twenty-four hours at a pH of 6.5 to 9.5. The use of iodine insures its complete conversion at any time. A high concentration of iodine results in loss of porphyrin. It also produces a tendency to slight emulsion formation. The recommended concentration does not have these disadvantages.

A study was made of the relative porphyrin concentrations obtained in duplicate analyses where the iodine wash preceded the sodium acetate washes in the first and followed them in the second analysis. Twenty-seven pairs of analyses were made of nine urines when freshly passed and again when kept in 0.1 and 1.0 per cent sodium carbonate solution for twenty-four hours. The mean ratios of the first to the second procedure were 0.96, 1.02, and 0.99, respectively. Variation about these means was minimal.

(g) *1.5N Hydrochloric Acid:*

A concentration of 1.5N hydrochloric acid was chosen for the following reasons. (1) Coproporphyrin is often present in urine as the zinc complex. This is more readily split and extracted from the ethyl acetate with 1.5N hydrochloric acid than with lower concentrations. (2) Higher concentrations of acid (i.e. 6 to 8N) will in some urines result in the formation of variable amounts of urorosein which tends to quench the porphyrin fluorescence. The stronger hydrochloric acids also removes other colored impurities left behind by the 1.5N hydrochloric acid.

8. *Comparison of the Improved Method With the Previously Used "Long" Method.*—

In a previous report[4] on the per diem excretion of coproporphyrin in normal individuals, a mean value of 54 μg per day was found. The "long" method, without carbonate preservative, was used for these analyses. On the other hand, data to be published indicate that the mean normal value in a comparable sample is approximately 160 μg per day when the improved method as now described is used. Factors responsible for loss of porphyrin or of fluorescence in the previously used "long" method have been defined in studies designed to isolate each of them. These factors may be considered under two main headings: (a) collection and preservation of urine and (b) analytic procedure.

(a) *Collection and Preservation of Urine:*

The mean coproporphyrin loss of 39 per cent obtained by omitting sodium carbonate during a twenty-four hour period of standing has been discussed already. It should be noted, however, that twenty-four hour collections of urine analyzed similarly soon after completion of the collection show a somewhat smaller loss in the absence of carbonate. This is due to the fact that the last specimens passed are exposed to deleterious conditions for only a fraction of the twenty-four hour period.

(b) *Analytic Procedure:*

(1) Conversion of precursor or chromogen: Recovery of coproporphyrin in the presence of appreciable amounts of precursor will vary depending on the time and speed of the analysis when iodine washes are omitted.[2] The analyses on which the previous per diem values were based were usually carried out two to six hours after completion of the twenty-four hour collection of urine. Determinations of chromogen and "native" porphyrin concentration were therefore carried out on thirty-two urines approximately four hours after completion of the twenty-four hour collection. No preservative was used. The improved method was employed in all cases, and the "long" method in addition in twenty-four. The amount of precursor obtained by the two methods was approximately the same, but considerably more "native" porphyrin was recovered with the new method. With the latter procedure the precursor accounted for an average of 19 per cent of the total (range 0 to 35 per cent), whereas with the "long" method it averaged 30 per cent of the total (range 0 to 48 per cent). Lack of a method for measuring precursor was therefore responsible in the previously reported analyses for an average coproporphyrin loss of 30 per cent.

In approximately 300 urines with or without carbonate preservative, which had stood at room temperature at various pH's for twenty-four hours, there was an average precursor concentration appreciably less than 1.0 μg per 100 milliliters. Ninety-nine per cent of these had less than 1.5 μg per 100 milliliters. Thus precursor is either converted to porphyrin or destroyed in this period.

(2) Extraction and fluorimetric analysis: A series of 329 urines was analyzed by both the improved and "long" methods. These urines were

preserved with carbonate. Analyses were made at least one day after completion of twenty-four hour collections, omitting the iodine washes. As already noted, there is little or no precursor remaining in such urines. Results are plotted in Fig. 5 in terms of the relative values obtained by the two methods. As shown in Fig. 5, a mean increase of 38 ± 25 per cent was obtained with the new method when compared with the "long" method.

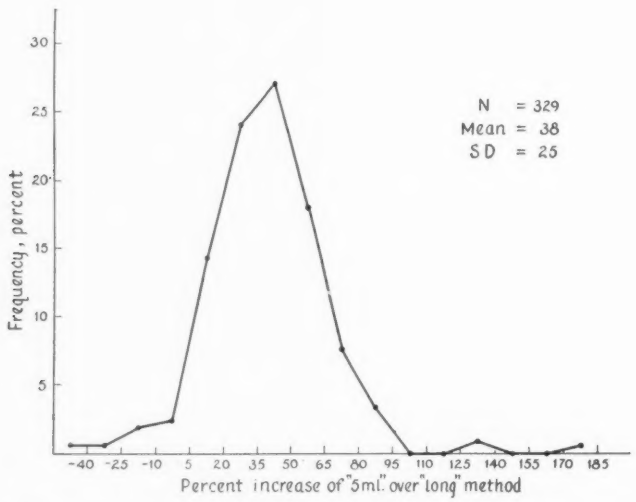

Fig. 5.—Distribution of comparative recoveries of urinary coproporphyrin by the "5 ml." and "long" methods.

The new method in the foregoing series employed a primary 405 mμ interference filter which transmits essentially monochromatic light. The "long" method, on the other hand, used a Corning filter No. 5-57 which transmits about 70 per cent at 365 mμ and 84 per cent at 405 mμ. Because of this difference, a second series of fifty-five urines was analyzed separately by both procedures. No carbonate was added to these urines, and the iodine wash was not used in the determination. Most of the samples were analyzed within five hours after completion of the twenty-four hour collection and so still contained variable amounts of precursor. The values obtained with the improved method were 43 per cent greater, on the average, than those with the "long" method (or, conversely, the latter showed a 30 per cent loss).

In order to separate losses due to differences in extraction from those due to differences in filters, the following comparisons were carried out. (a) The fluorescence intensities of the extracts obtained with the new and "long" methods were compared using the same 405 mμ interference filter, thus giving a direct analysis of the relative extraction efficiencies alone. An average loss of 17 per cent was observed with the "long" method. (b) The extracts obtained with the "long" method were read first with the 405 mμ interference filter and then with the Corning No. 5-57 filter, giving a direct evaluation of the role of primary filters alone. Readings with the Corning filter were, on the average, 13 per cent

lower. It would appear, therefore, that in the original comparison of the improved and "long" methods 17 per cent of the loss with the latter method was due to less efficient extraction and 13 per cent to imperfect fluorimetric analysis.

It should also be noted that the 38 per cent increase, obtained with the new method on urine samples preserved with carbonate and in which essentially no precursor remained, is insignificantly different from the 43 per cent increase obtained with samples having variable pH's and containing variable amounts of precursor. It is therefore apparent that this increase is relatively independent of urine pH or amount of precursor.

As previously indicated in Table IV, considerable latitude is offered in the choice of primary filters with the new method. This was further illustrated in twenty urines analyzed first with the Corning filter No. 5-57 and then with the 405 mμ interference filter. An insignificant mean difference of 1.2 per cent was found. The lack of difference here as compared with that noted for the "long" method is believed to be due to greater dilution of quenching materials with the improved method.

(c) *Summary of Factors Responsible for Porphyrin Loss With the "Long" Method:*

The factors accounting for the differences in results between the present improved method and the previously used "long" method are (1) pH control, (2) precursor conversion, (3) extraction efficiency, and (4) fluorimetric efficiency.

Control of pH yielded a nearly neutral or alkaline urine which is necessary to prevent porphyrin loss. Failure to control pH resulted in an average loss of 39 per cent.

Conversion of precursor to porphyrin may take place while the alkaline urine is standing or may be accomplished during the extraction procedure. Failure to recover the precursor resulted in an average loss of 30 per cent.

The efficiency of porphyrin extraction in the improved method is greater because of the relative simplicity of the analytic procedure, the use of relatively large volumes of ethyl acetate in place of ether, and the use of buffered acetic acid in place of glacial acetic acid. The less efficient "long" method resulted in an average extraction loss of 17 per cent.

The efficiency of the fluorimetric measurement varies with the purity of the exciting light. Use of nonspecific wave lengths results in extraneous fluorescence and in added quenching effects. The less selective filter of the "long" method resulted in an average porphyrin loss of 13 per cent.

The over-all contribution of these factors to the total porphyrin loss is summarized in Table VI in terms of their absolute and per cent loss, as well as the cumulative absolute loss with each successive step of the analytic procedure. This analysis is based on a mean normal urinary coproporphyrin of approximately 160μg per day using the improved method. The average cumulative loss is 108 μg per day. On the basis of these calculations the average per diem coproporphyrin value expected with the "long" method in a sample of normal subjects comparable to that previously reported is 52 μg per day (160–108). The value actually reported was 54 μg per day.

TABLE VI. AVERAGE COPROPORPHYRIN LOSS IN THE PREVIOUSLY USED "LONG" METHOD WHEN COMPARED WITH THE IMPROVED METHOD

SOURCE OF LOSS	COPROPORPHYRIN LOSS*		CUMULATIVE LOSS (μG/DAY)
	%	μG/DAY	
1. Lack of pH control	39	62	62
2. Loss of precursor	30	29	91
3. Incomplete extraction	17	12	103
4. Inadequate fluorimetry	13	5	108

*Based on a mean value of 160 μg per day with the improved method. The sources of loss tabulated follow the sequence of the analytic procedure. The absolute loss at each step is calculated from the residual coproporphyrin remaining at that point in the procedure.

SUMMARY

An improved and simplified method for determining urinary coproporphyrin is presented. Construction of a sensitive fluorimeter, which is desirable for this determination, is described. The use of a comparatively small ratio of urine volume to final extract volume makes possible increased accuracy and a wider choice of primary and secondary filters. An efficient combination of filters and their transmission characteristics are listed.

Fluorescence of coproporphyrin is enhanced by an average of 8 per cent when hydrochloric acid extracts are saturated with ethyl acetate or ether. The incorporation of a correction for this effect in preparing standards is noted.

Analyses are reproducible within an error of 3 to 4 per cent.

Evidence is presented to indicate that the measured fluorescence of the final hydrochloric acid extract is due entirely to coproporphyrin.

Addition of sodium carbonate to the urine as a preservative over a twenty-four hour period results in an average increase of 65 per cent in the amount of coproporphyrin recovered. Complete recovery of coproporphyrin is obtained in urines kept at pH 6.5 to 9.5. In a neutral or alkaline urine the coproporphyrin concentration is reasonably stable.

Coproporphyrin precursor or chromogen is nearly all converted to porphyrin on standing twenty-four hours in neutral or alkaline urine. In acid urine it is largely destroyed over the same period. The use of an iodine wash is recommended to convert the precursor remaining at any given time after completion of the collection.

With the apparatus and filter combinations recommended, fluorescence intensity is linearly related to porphyrin concentration below 5 μg per cent. At higher concentrations there is increasing departure from linearity, maximum readings being obtained at 140 μg per cent. Beyond this point there is marked diminution of fluorescence because of light absorption by the porphyrin. Maximum fluorescence with the mercury arc lamp is excited by 405 mμ light. The shape of the calibration curve varies with the wave length of the exciting light.

Coproporphyrin fluorescence decreases with increasing hydrochloric acid concentration. The relative loss of fluorescence is greater as the coproporphyrin concentration increases in the range of 5 to 100 μg per cent. Fluorescence intensity decreases as the temperature rises in the range of 10° to 50° C.

The reasons for selecting the reagents, volumes, and concentrations employed in the improved method are reviewed.

The sources of porphyrin loss in a previously used "long" method for the determination of urinary coproporphyrin are quantitatively defined as lack of pH control, loss of precursor, incomplete extraction, and imperfect fluorimetry.

The authors are indebted to Catherine Evertz and to Dr. Robert Aldrich for their contributions in the beginning phases of this investigation. They also wish to recognize the technical assistance of Audrey Christenson, Jean Gannon, Mary Graves, Rebecca Hackner, Shirley Hill, Irene Bossenmaier, and Violet Hawkinson.

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ACETYLCHOLINE AND CHOLINESTERASE IN THE BLOOD OF PATIENTS SUFFERING WITH BRONCHIAL ASTHMA

HAROLD H. SCUDAMORE, M.D., PH.D.,* LOUIS J. VORHAUS II, M.D.,** AND ROBERT M. KARK, M.R.C.P. (LOND.), F.A.C.P.**
CHICAGO, ILL.

ONE hypothesis, which has been advanced to explain the pathologic physiology of bronchial asthma, suggests that an imbalance of the autonomic nervous system may play a role in the disease. The autonomic imbalance could be produced by decreased activity in the cholinesterase enzyme system, by increased parasympathetic activity, or by other alterations which might result in liberation or production of increased quantities of acetylcholine in the end organs or in the blood flowing through the lungs.

Eppinger and Hess,[9] in 1909, were among the first to propose that bronchial asthma was an example of pathologic increased vagotonia. Since then a large body of indirect evidence has been collected which suggests a close relationship may exist between acetylcholine activity in the body and attacks of asthma. These data have been summarized by Miscall and Rovenstine,[16] Curry,[5] and Beakey and co-workers.[3] As far as we are aware, excessive amounts of acetylcholine have never been demonstrated in the blood, respiratory tissues, or at the bronchial nerve endings of patients ill with asthma, nor has it been established that the cholinesterase activity of the blood or tissues is altered in bronchial asthma. Milhorat[15] found normal values for serum cholinesterase in patients with bronchial asthma, and Hawes and Alles[12] measured whole blood cholinesterase and found this to be normal in asthmatic patients, even during attacks of asthma.

In this communication observations on serum acetylcholine levels and on plasma and erythrocyte cholinesterase activities in healthy individuals and in patients ill with bronchial asthma are reported.

METHODS

Patients and Healthy Subjects.—Venous blood was obtained for study from fourteen volunteer healthy individuals between the ages of 20 and 57 years and from fourteen patients suffering with asthma. The patients were seen either in the wards or in the allergy clinic of the Research and Educational Hospital, University of Illinois. Each patient was classified on the basis of etiology, severity of the asthma, duration of symptoms, and the severity of symptoms at the time the blood specimen was obtained (graded from + to ++++). As shown in Table II, twelve of the patients were considered to have predominantly extrinsic asthma; four of these suffered with seasonal allergic asthma and eight with perennial asthma. In some patients the disease was complicated by emotional factors or by bronchitis. In two patients the predominant etiologic factor was considered to be intrinsic in nature.

From the Army Medical Nutrition Laboratory, an installation under the jurisdiction of the Office of the Surgeon General, United States Army, and the Department of Medicine, University of Illinois College of Medicine.

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*Present address: Mayo Foundation, Rochester, Minn.

**Present address: Department of Medicine, University of Illinois College of Medicine, Chicago, Ill.

Acetylcholine Assay.—The total of acetylcholine-like substances in the serum was determined using the isolated heart of the clam, *Venus mercenaria*, as suggested by Wait.[27] The details of the method of obtaining and preserving the serum specimens with Prostigmine and of the procedure for making the determinations are described elsewhere.[21] Examples of kymograph tracings obtained in the acetylcholine assays are illustrated in Fig. 1. The results are expressed as micrograms of acetylcholine per 100 ml. of serum (mcg.%).

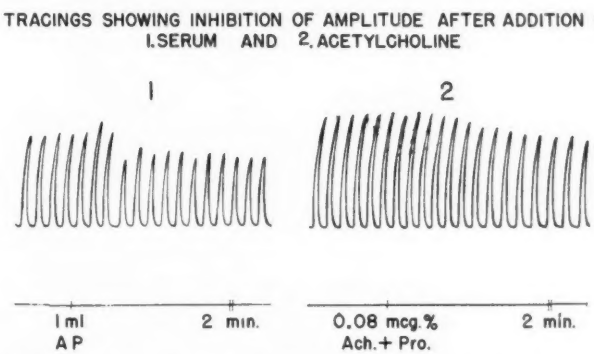

Fig. 1.—Kymograph tracings of acetylcholine assay. *1*, Tracing showing 24 per cent inhibition in amplitude of clam heartbeat two minutes after addition to heart chamber of 1 ml. of patient's serum filtrate (*AP*) at a dilution of 1:2. *2*, Tracing indicating 23 per cent inhibition two minutes after the addition of acetylcholine chloride solution containing Prostigmine methyl sulfate (*Ach. + Pro.*). The final concentration of this solution was 0.08 μg per 100 ml. with respect to acetylcholine.

The various substances in human serum which are most likely to interfere with the test were without significant effect in the studies made by Tower and McEachern.[24] Rose[20] has shown that histamine in very high concentrations does not effect the clam heart. The Prostigmine used as a preservative has no significant effect on the amplitude of the heartbeat.[21] Welsh and Taub[28] have shown that choline and esters other than acetylcholine will depress the amplitude of the clam heartbeat, but only in concentrations of 80 to 15,000 times greater than that of acetylcholine. Because all the substances already described may affect the clam heart, it is more accurate to describe the assay as being one for "acetylcholine-like substances." In this report, then, although we believe we have measured only acetylcholine, the term "acetylcholine" will be used to include acetylcholine-like substances.

Cholinesterase Determinations.—The plasma and erythrocyte cholinesterase activities were measured by the electrometric method of Michel[14] as described previously.[22, 26] According to this method, cholinesterase activity is expressed as the decrease in pH units per hour (△ pH/hr.).

RESULTS

Healthy Individuals.—The results of determination of serum acetylcholine and plasma and erythrocyte cholinesterase activities in fourteen healthy persons are shown in Table I. The mean value for the acetylcholine-like substances in the serum was 1.28 ± 1.20* μg per 100 milliliters. There was a wide variation of values from 0.32 to 4.80 μg per 100 ml. of serum. The normal range would, therefore, have to be considered from 0 to 3.68† μg per 100 ml. of serum. Healthy Subject M. E., in whom there was found the highest value of acetylcholine, had some spasm of the duodenum by fluoroscopy but a positive diagnosis of ulcer was never made.

*Mean ± standard deviation.
†Mean ± two standard deviations.

TABLE I. SERUM ACETYLCHOLINE LEVELS AND BLOOD CHOLINESTERASE ACTIVITY OF HEALTHY INDIVIDUALS

SUBJECT	AGE	SEX	SERUM ACETYLCHOLINE (μG/100 ML.)*	CHOLINESTERASE ACTIVITY (△ pH/HR.)	
				PLASMA	ERYTHROCYTE
D. B.	26	M	0.62	0.61	—
C. B.	28	M	0.35	1.04	0.64
F. C.	44	M	0.80	1.14	0.68
M. E.	20	M	4.80	1.02	0.79
R. G.	34	F	0.40	0.70	0.63
R. H.	24	F	1.20	0.88	0.60
T. H.	25	M	2.75	0.95	0.69
S. K.	47	F	1.86	1.08	0.58
E. M.	27	M	0.80	1.04	0.65
M. M.	32	F	0.91	0.76	0.92
M. Y.	57	F	1.20	1.31	0.56
V. S.	29	F	0.80	0.77	0.68
H. S.	33	M	0.30	0.95	0.70
W. T.	25	M	1.13	0.92	0.59
Mean			1.28	0.94	0.67
Standard deviation			±1.20	±0.19	±0.10
Standard error			±0.32	±0.05	±0.03

*μg = micrograms.

The mean plasma cholinesterase activity of the healthy group was 0.94 ± 0.19 △ pH per hour, which is essentially the same as that reported by us[26] for sixty-eight healthy subjects. The mean erythrocyte cholinesterase activity was 0.67 ± 0.10 △ pH per hour as compared to a mean of 0.71 ± 0.11 △ pH per hour reported in a larger series of healthy subjects.[22]

Patients Ill With Bronchial Asthma.—The results of determinations of acetylcholine in serum from eight patients ill with bronchial asthma, together with data on plasma cholinesterase activity in fourteen and erythrocyte cholin-

TABLE II. SERUM ACETYLCHOLINE LEVELS AND BLOOD CHOLINESTERASE ACTIVITY IN PATIENTS ILL WITH BRONCHIAL ASTHMA

PATIENT	AGE	SEX	MAIN ETIOLOGIC FACTORS			SEVERITY	DURATION (YR.)	CONDITION OF PATIENT*	SERUM ACETYLCHOLINE (μG %)†	CHOLINESTERASE ACTIVITY (△ pH/HR.)	
			EXTRINSIC		INTRINSIC					PLASMA	ERYTHROCYTE
			SEASONAL	PERENNIAL							
M. P.	38	F	x			Severe	14	++++	3.95	0.89	0.25
G. P.	64	M			x	Severe	9	++++	2.60	1.25	—
M. N.	39	M	x			Severe	32	++	6.40	1.20	0.79
M. O.	41	F			x	Severe	2	+++	3.48	0.72	—
A. P.	51	F		x		Moderate	3	++	3.28	0.96	—
V. H.	38	F		x		Severe	6	+++	3.00	0.88	—
I. M.	39	F		x		Severe	35	+++	3.04	1.13	—
S. R.	43	F		x		Mild	30	+++	6.00	1.22	0.70
E. H.	64	M		x		Mild	10	+++	—	0.85	0.65
F. T.	45	F		x		Mild	3	++	—	0.92	0.71
I. P.	40	F	x			Severe	—	—	—	0.78	0.97
W. J.	33	M	x			Mild	19	+	—	0.66	0.70
H. M.	42	F		x		Severe	19	++	—	0.85	—
F. Z.	25	F		x		Mild	1	+	—	1.10	—
Mean									3.97	0.96	0.68
Standard deviation									±1.44	±0.10	±0.2
Standard error									±0.51	±0.06	±0.0

*Severity of asthmatic symptoms when blood sample was obtained.
†μg % = Micrograms per 100 ml. of serum.

esterase activity in seven patients, are shown in Table II. Patient M. P. was having severe asthmatic symptoms when the blood specimen was taken, and the blood specimen was obtained from Patient G. P., who was also having a severe attack of asthma, a few minutes after 0.5 ml. of epinephrine (1:1,000) had been injected subcutaneously.

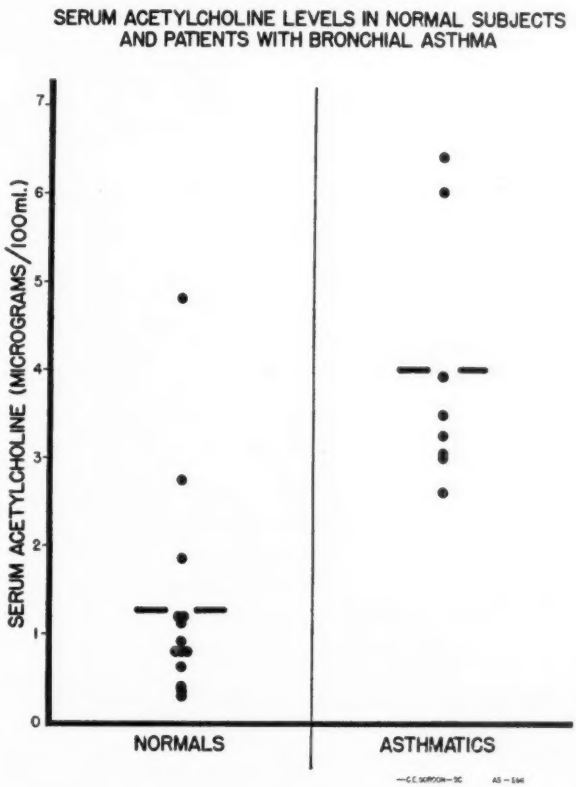

Fig. 2.—Comparison of the serum acetylcholine levels in fourteen healthy men and women with eight patients suffering with bronchial asthma. The horizontal bars represent the mean values.

The mean serum acetylcholine in the asthmatic patients was 3.97 ± 1.44 μg per 100 milliliters. A scattergram comparing the range of values in the asthmatic patients and healthy subjects is shown in Fig. 2. There is considerable variation and overlap in the two groups. Half of the values are above the upper limit of the normal range and the remainder are high normal levels, but all are above the normal mean. The difference of the means between the healthy group and the patients ill with asthma was 4.5 times greater than the standard error of the difference and, statistically, this is highly significant.[13] The two patients in severe attacks of asthma had intermediate serum acetylcholine levels. Although the number of observations is too small for statistical analysis, by simple inspection of the data there does not seem to be any correlation between severity of asthma and level of acetylcholine.

The mean plasma cholinesterase activity of fourteen asthmatic patients was 0.96 ± 0.10 △ pH per hour, which was essentially the same as that found in the fourteen healthy individuals. The mean erythrocyte cholinesterase activity was 0.68 ± 0.22 △ pH per hour, which was also essentially the same as that found in thirteen healthy individuals. An extremely low erythrocyte cholinesterase was found in one patient (M. P.) from whom a specimen of blood was obtained during a severe attack of asthma. This patient was not ill with a blood dyscrasia or other disease which might produce a low level of erythrocyte cholinesterase activity.

Changes During a Severe Asthmatic Attack.—One patient (M. P.) was observed during a severe attack of asthma. Samples of blood were obtained for analysis at the peak of the attack and fifty minutes after the subcutaneous administration of 0.3 ml. of epinephrine (1:1,000) when most of the symptoms had subsided. The results are shown in Table III. All of the changes were minimal and, probably, are not significant. The most interesting observation was the slight increase in the abnormally low erythrocyte cholinesterase activity and concomitant slight decrease in the serum acetylcholine level following therapy.

TABLE III. CHANGES IN SERUM ACETYLCHOLINE AND BLOOD CHOLINESTERASE ACTIVITY IN A PATIENT DURING A SEVERE ASTHMATIC ATTACK

CONDITION	SERUM ACETYLCHOLINE (μG/100 ML.)	CHOLINESTERASE ACTIVITY (△ pH/HR.)	
		PLASMA	ERYTHROCYTE
During asthmatic attack	3.95	0.89	0.25
50 min. after 0.3 ml. 1:1,000 epinephrine	3.64	0.72	0.35

DISCUSSION

There is considerable indirect evidence supporting the hypothesis that autonomic dysfunction may occur in the asthmatic patient. For example, the administration of Prostigmine,[11] eserine,[8] or acetyl-beta-methylcholine (Mecholyl)[3, 5, 17, 23, 25] may produce an asthmatic attack or reduce the vital capacity in asthmatic patients, but has no such effects in healthy subjects. Moreover, it has been shown also[3, 6] that Prostigmine potentiates the effect of Mecholyl, and that anticholinergic and adrenergic drugs protect against the Mecholyl-induced "asthma."

Other observations do not support the autonomic imbalance hypothesis. For instance, anticholinergic drugs, such as atropine, alone will not control the asthmatic attack. In addition, it is well known that the surgical treatment of intractable asthma by resection of the parasympathetic nerve supply to the bronchial tree has not been entirely successful.[4, 10] Finally, Musser and Grimm,[18] using the rabbit intestine as a test object, found that the serum from asthmatic patients produced adrenergic responses more often than cholinergic ones.

The observations reported in this paper of significantly increased levels of acetylcholine in the serum from eight patients ill with asthma must not be

interpreted as evidence that parasympathetic overactivity is the cause of asthma. It is equally possible that the increased acetylcholine levels are the result of asthma. While exact interpretation of the data must await further investigation it is interesting to note that Wenner and Buhrmester[29] found relatively large quantities of acetylcholine in the blood of rabbits in anaphylactic shock as compared with none in the blood from control animals.

With regard to the cholinesterase activity of the blood, the results herein reported are in agreement with previous reports[12, 15] that there is no significant difference between the plasma or erythrocyte cholinesterase activity of healthy individuals and patients ill with bronchial asthma. Therefore, the increased amounts of acetylcholine in serum are not due to reduced cholinesterase activity of the blood. Alterations in the cholinesterase enzyme system probably do not play an important role in the pathologic physiology of asthma.

The results reported in this paper suggest the need for further studies of autonomic nervous mechanisms that may be involved, along with many other factors,[19] in the causation of asthma, and of the effects of anticholinergic drugs in therapy in this disease. They also offer a rational explanation for the dangerous effects of morphine, a cholinergic drug, when used to treat patients ill with bronchial asthma.

SUMMARY

The results of determination of serum acetylcholine levels and of plasma and erythrocyte cholinesterase activity in healthy individuals and in patients suffering with bronchial asthma are reported.

The mean acetylcholine level in serum from eight patients suffering with asthma was significantly greater than the mean value for healthy subjects.

Plasma and erythrocyte cholinesterase activity was found to be normal in blood taken from fourteen patients ill with asthma.

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ARTIFACTUAL VALUES OF SERUM PRECIPITABLE IODINE AFTER CLINICAL INTRAMUSCULAR INJECTIONS OF MERCUHYDRIN

JOHN H. MEYERS, M.D.,* AND EVELYN B. MAN, PH.D.
NEW HAVEN, CONN.

SPURIOUSLY high values for serum precipitable iodine (SPI) have been reported for patients who had received for diagnostic purposes certain organic compounds containing iodine.[1, 2] This investigation is concerned with the occurrence and duration of falsely low values of SPI in patients who received Mercuhydrin (sodium salt of methoxy-oxymercuripropylsuccinylurea) for diuretic purposes.

SUBJECTS AND METHODS

The data are presented in Table I. Of the twelve patients, the first nine had heart failure, complicated in the first two by hyperthyroidism. Only one of these two, C3622, was receiving iodine and propylthiouracil. The last three patients had Laënnec's cirrhosis of the liver. All twelve had edema. None had received within a significant period any of the iodine compounds used as contrast media in roentgenography which give false high values.[1, 2]

All patients received 1 or 2 c.c. of Mercuhydrin containing, according to Modell, Gold, and Clarke,[3] 0.039 Gm. of Hg per cubic centimeter. SPI was determined, whenever possible, before, one or two hours after injection of the drug, and one to six days later.

SPI was measured by the method of Man.[4] Duplicate analyses which agreed within 1 gamma per cent were averaged. In some instances agreement between duplicates or quadruplicates was so unsatisfactory that values could not be averaged. This rarely occurs in ordinary determinations. Such a discrepancy appears in the pretreatment determination in Case 23797. Similar and larger discrepancies occurred, however, in five of the analyses after Mercuhydrin.

RESULTS

It is evident from the table that one or two hours after intramuscular injections of Mercuhydrin, values for SPI are reduced. In five instances they had fallen to 1.7 to 3.3 gamma per cent, figures usually found in hypothyroid patients.

SPI was measured before and twenty-four hours after Mercuhydrin in eight instances. In six of these the concentrations before and after this interval differed from one another by no more than 1.0 gamma per cent, the degree of variation observed from day to day in normal subjects.[4] All of these patients excreted adequate amounts of urine. In Case A21619 SPI had returned to its original value in forty-eight hours. In three cases, 23797, C36067, and B97358, SPI was still low after twenty-four hours. In the first and last of these Mercuhydrin had no diuretic effect. No record of weight was obtained on Case C36067.

From the Department of Internal Medicine, Yale University School of Medicine.
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TABLE I. THE EFFECT OF MERCUHYDRIN ON SERUM PRECIPITABLE IODINE (SPI)

CASE	AGE	SEX	INTERVAL* (DAYS)	DATE	TIME (A.M.)	MERCUHYDRIN (C.C.)	WEIGHT (KG.)	SPI (γ %)
A51665	46	F	60	1/19	9	2		8.8
					10			3.3
				1/20				9.0
C3622	57	M	5	3/1	9	2	46.9	12.3
					10			4.1
				3/2	9		46.1	11.3
				3/4	9			9.5
81644	59	M	2	1/25	9	2		8.0
					10			2.2
				1/26	9			8.9
23797	72	M	2	1/18	9	2	63.4	8.7, 10.9
					10			2.3
				1/19	9		63.2	7.6, 5.3
				1/24	9		63.0	8.0
38426	77	M	3	1/30	9	2	65.7	6.9
					10			4.5, 6.0, 1.1
				1/31	9		65.8	7.3
				2/1	9		64.3	7.2
B30265	58	M	1	2/1			63.8	
				2/2	9	1		4.4
					10			5.1, 3.1, 3.7, 2.6
				2/3	9		63.2	4.9
A21619	79	M	3	1/30	9	2	60.2	5.7
					10			1.7
				2/1	9		59.0	5.8
C36067	66	M		5/25	9	2		
				5/26	9			1.6, 2.9
				6/2	9			4.9
B97358	72	F	1	1/9	9	1	52.5	1.8
					9:30			1.9
					10			2.3
				1/10	9	1	52.5	5.1
				1/11	9	1	52.3	3.1
B89399	56	M	2	1/23	9	1	53.8	8.8
					11			7.4, 7.4, 3.7, 2.6
				1/24	9		53.2	8.8
				1/27	9			8.6
C39255	68	M	1	3/23	8	2	71.8	
					10			1.8
				3/29	9		68.2	5.6
A27691	64	M	7	12/13	9	2		4.9
					11			5.1

*The number of days since the last injection of Mercuhydrin.

DISCUSSION

In acutely ill cardiac patients, SPI is especially important in the detection of disorders of thyroid activity because measurements of basal metabolism are unsatisfactory. The disturbing effect of mercurial diuretics, therefore, is of great significance. So irregular is this effect that no correction can be made for it. In this series of cases Mercuhydrin lowered SPI by amounts varying from 4.0 to 8.2 gamma per cent.

The false low values can be attributed to the formation of insoluble mercuric iodate or iodide during the technical processes of digestion in consequence of which iodine is not distilled. If a thermometer breaks during digestion or distillation, no iodine is recovered in the distillate.

The duration of the interfering effect of Mercuhydrin depends on the rate of excretion of the mercury. In most subjects this appears to be practically complete in twenty-four hours. Milnor and associates[5] found a maximum concentration of radio-mercury in the urine twenty to sixty minutes after intravenous injection of the tagged compound. The concentration in the blood had decreased to 20 per cent or less 100 minutes after the injection. In certain patients, however, excretion appears to be delayed. In Cases B97358 and C36067 values of 1.8 and 1.6 gamma per cent were observed twenty-four hours after injection of 1 and 2 c.c. of drug respectively. Subsequent concentrations were 5.1 and 4.9 gamma per cent. These patients had no diuresis. Presumably excretion of mercury was delayed. On the other hand, in Case A27691, who noted an unusually large diuresis in the first thirty minutes after injection of 2 c.c. of the drug (unfortunately measurements of weight and urinary output were not obtained), SPI after only two hours coincided with its initial concentration. Because of this variability in the rate of excretion of mercury, reliance can not be placed on measurements of SPI within forty-eight hours of an injection of Mercuhydrin. In some cases it may be necessary to allow an even longer period for the elimination of the mercury.

Although these experiments have dealt only with the effect of intramuscular injections of Mercuhydrin, they are presumably relevant to mercurial diuretics in general.

CONCLUSIONS

After intramuscular injection of Mercuhydrin, SPI drops sharply. This drop is referable not to any diminution of the quantity of iodine attached to the proteins of the serum, but to the formation of insoluble mercuric iodate and iodide in the analytic procedure. The interfering effect of the mercury is usually dissipated within twenty-four hours, but in some patients, because excretion of mercury is delayed, it may persist longer.

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OBSERVATIONS ON THE EFFECT OF CORTISONE ACETATE ON TWO PATIENTS WITH HEPATIC DISEASE

HUGH R. BUTT, M.D.,* MANDRED W. COMFORT, M.D.,* MARSCHELLE H. POWER, PH.D.,** AND HAROLD L. MASON, PH.D.**
ROCHESTER, MINN.

THAT the liver is intimately associated with the metabolism of many of the hormones of the body is well established. It has been assumed by many that hepatic damage results in an inability of the organ to metabolize certain hormones properly and that under such circumstances physiologic activity is altered. The purpose of this communication is to describe some results that were noted following the administration of cortisone acetate to two patients with hepatic parenchymal damage.

Patient S, who had primary biliary cirrhosis, received 100 mg. of cortisone acetate twice daily intramuscularly as a saline suspension of finely ground crystals containing 25 mg. per cubic centimeter for a period of twelve days. Patient G, who had serum hepatitis and cirrhosis, received 50 mg. of cortisone twice daily for one period of fourteen days and for a second period of five days.

The patients were maintained in the metabolic study unit of the Mayo Clinic, where balance data and other laboratory data were collected before, during, and after the periods of administration of cortisone. The procedure and methods employed are given in detail in the article by Sprague and associates.[1] The metabolic data are recorded for purposes of comparison with responses that may be noted by others in future studies of hepatic disease.

REPORT OF CASES

CASE G.—A 49-year-old man was admitted to the clinic Sept. 7, 1949, complaining of progressive rheumatoid arthritis, which had been present for twelve years. Most of the joints were involved, including the jaws, neck, feet and hands, back, hips, and shoulders. While undergoing his examination he noted dark urine and a few days later scleral icterus. At this time a history was obtained that the patient had received one unit of Red Cross blood plasma in March, 1949, and frequent injections of streptomycin for several weeks prior to June, 1949. This history with physical and laboratory examinations made the diagnosis of homologous serum hepatitis most likely. It was noted that several spider angiomas were about his face, and that palmar erythema and fetor hepaticus were present. A roentgenogram of the thorax showed bilateral symmetrical pulmonary fibrosis (the patient had been a coal miner for several years), and multiple roentgenograms of the joints showed destructive arthritis and osteoporosis of the bones. A transthoracic needle biopsy of the liver obtained on Sept. 28, 1949, was compatible with chronic hepatitis and early cirrhosis.

Two weeks later the total bilirubin concentration was 28 mg. per 100 c.c. of serum and at this time administration of 50 mg. of cortisone acetate twice daily intramuscularly was begun. This dosage of cortisone acetate was continued for fourteen days. There followed an interval of thirty days during which the serum bilirubin became nearly normal and during

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*Division of Medicine, Mayo Clinic.
**Division of Biochemistry, Mayo Foundation.

which no cortisone was administered. Then with a relapse of the hepatitis the cortisone was given in the same dosage for a period of five days followed shortly thereafter by death from ruptured esophageal varices and hepatic coma.

CASE S.—A 20-year-old woman, mother of three normal children, had a family history and childhood which were essentially normal. In 1933 she had had a simple appendectomy. In the early part of her third pregnancy in July, 1948, she noted some anorexia and occasional nausea and easier fatigability than had been present with her other pregnancies. She had a premature delivery in January, 1949, at seven months. It was in the middle of June, 1949, that a friend first noted her scleras to be icteric. The icterus had persisted to date without much variation. About the same time she began to notice a mild generalized pruritus which had persisted to date. She was first admitted to the clinic Dec. 5, 1949. At that time she was jaundiced and there were xanthomatous skin deposits over the elbows, back, hands, and surfaces of the arms. The liver and spleen could not be palpated. There was no fetor hepaticus, rubor, or spider angiomas. She complained of pain in her joints, worse on motion and relieved by rest. Roentgenograms of the thorax and joints were all normal. Needle biopsy obtained Dec. 9, 1949, showed a microscopic picture typical of biliary cirrhosis.

She returned again on Jan. 18, 1950, for metabolic study.

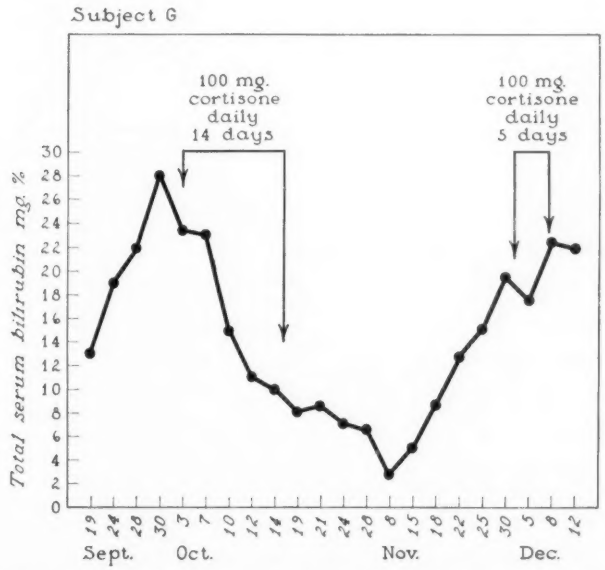

Fig. 1.—Concentration of bilirubin in the serum of Patient G before, during, and after administration of cortisone.

CLINICAL OBSERVATIONS

CASE G.—As the bilirubin rose at the onset of illness to about 12 mg. per 100 c.c. of serum, the patient noted about 75 per cent relief of his painful rheumatism and he was able to move about with ease. Ten to 20 per cent additional relief was noted during administration of cortisone.

During the administration of cortisone in this period of fourteen days the patient was quite comfortable; there was slight edema that developed at the ankles and for the first time a slight amount of fluid was noted in the abdomen. No changes were noted in the spider angiomas, the fetor hepaticus, or the palmar erythema which was present in this patient, and there was no change in size of the liver during the administration of cortisone. There was no particular improvement in appetite or any subjective sense of well-being except relief

TABLE I. LABORATORY DATA OF PATIENT WITH CIRRHOSIS SECONDARY TO SERUM HEPATITIS RECEIVING CORTISONE (SUBJECT G)

TIME (1949)	THYMOL TURBIDITY	$ZnSO_4$ TURBIDITY	CEPHALIN-CHOLESTEROL FLOCCULATION	TOTAL CHOLESTEROL (MG. %)	CHOLESTEROL ESTERS (MG. %)	ALKALINE PHOSPHATASE (UNITS)	PROTHROMBIN TIME (SEC.)	ALBUMIN (GM.)	GLOBULIN (GM.)	HEMOGLOBIN (GM.)	ERYTHROCYTES*	LEUCOCYTES†	SEDIMENTATION RATE (MM. PER HR.)
9/ 8								3.7	3.8	14.1	4.11	4.8	55
9/19	17	38	3+	167	152	7.9	21						65
9/30	15	34	3+	117	22		19	2.8	3.9	13.4	4.14	6.9	80
10/ 3‡	13	30	3+									6.2	77
10/10	11	28	2+	107	20		22					4.7	79
10/17	16	23	3+	180	62		19	3.1	3.2			8.5	65
10/24	11	23	3+	217	127	6.5		3.3	2.9	11.9			75
11/15										14.0	3.9	6.5	96
11/25	19	43	3+	123	29		19	2.5	4.0			6.6	106
11/30	19	53	3+				21			13.4		4.1	102
12/ 2§												8.0	98
12/ 5												6.6	83
12/ 7												21.3	64
12/ 8	15	43	3+	87	20		21	2.3	4.4			16.0	76

*Millions per cubic millimeter of blood.
†Thousands per cubic millimeter of blood.
‡From Oct. 3, 1949, through Oct. 16, 1949, given 100 mg. cortisone acetate daily.
§From Dec. 2, 1949, through Dec. 7, 1949, given 100 mg. cortisone acetate daily.

of the arthritic pain. The bilirubin fell from 28 mg. to 10 mg. per 100 c.c. of serum in a period of about ten days (Fig. 1). We have seen many other cases of severe hepatitis, however, in which the bilirubin concentration has fallen just as rapidly without cortisone.

As noted in Table I the blood sedimentation rate fell during administration of cortisone; the flocculation reactions for hepatic function were unaltered; cholesterol and cholesterol esters fell as the disease progressed and returned toward normal as improvement was noted. The serum protein was changed but little except for the usual fall in serum albumin noted in severe hepatic disease. In Table II the electrolyte changes noted were of little significance except for slight retention of sodium during administration of cortisone.

TABLE II. ELECTROLYTES IN SERUM (OR PLASMA) OF PATIENT WITH CIRRHOSIS SECONDARY TO SERUM HEPATITIS RECEIVING CORTISONE (SUBJECT G)

TIME (1949)	SODIUM (MEQ./L.)	POTASSIUM (MEQ./L.)	CHLORIDE (MEQ./L.)	CARBON DIOXIDE (MEQ./L.)	CALCIUM (MG. %)	PHOSPHORUS (MG. %)
9/24	138	3.6				
9/30*	129	3.3	105	23.2	8.9	2.8
10/ 7	136	4.1	108	24.0		
10/10	135	3.5	107	21.8		
10/17	139	4.4	108			3.2
10/24	141	4.3	104	23.0		
11/21	136	3.8	101	23.0		
12/ 5†	129	3.9	105	19.6		
12/12	136	3.2	94	20.5		

*From Oct. 3, 1949, through Oct. 16, 1949, given 100 mg. cortisone actetate daily.
†From Dec. 2, 1949, through Dec. 7, 1949, given 100 mg. cortisone acetate daily.

Over the next four weeks the bilirubin level fell nearly to normal. The ascites and most of the edema disappeared. The patient's stiffness and joint pain slowly returned and then gradually the concentration of bilirubin began to increase, and ascites, edema, and signs and symptoms of a severe postnecrotic cirrhosis developed. As the level of the serum bilirubin concentration rose there was only about a 25 per cent relief of rheumatic symptoms, and the addition of cortisone, which was started at the same dosage as before, did not have any noticeable effect on the rheumatic symptoms. The patient at this time was very ill, he became drowsy, ascites and edema rapidly developed, and he began vomiting coffee ground material. After five days, administration of cortisone was discontinued because the patient was much worse clinically. A few days after administration of cortisone had been discontinued the patient had a very severe gastrointestinal hemorrhage, lapsed into coma, and died shortly thereafter. Necropsy disclosed severe postnecrotic cirrhosis with esophageal varices and some ulceration of the stomach.

CASE S.—This patient received 200 mg. of cortisone acetate daily for two consecutive periods of six days each. During this period of therapy there was no change in any of the physical findings. Subjectively the patient noted less discomfort in her joints and some insomnia.

TABLE III. PLASMA LIPIDS DURING CORTISONE THERAPY (SUBJECT S)

TIME	CHOLESTEROL (MG. %)	CHOLESTEROL ESTERS (MG. %)	FATTY ACIDS (MG. %)	TOTAL LIPIDS (MG. %)
12/ 5/49	1,788		3,512	5,300
1/21/50	1,412	143	3,400	4,812
1/28/50	1,532	114	3,768	5,300
2/ 3/50*	1,872	126	2,517	4,384
2/ 9/50	1,624	140	2,920	4,544
2/15/50	1,736	224	3,360	5,096
2/21/50	1,976	320	2,672	4,648
2/27/50	1,736	372	2,616	4,352

*From Feb. 3, 1950, through Feb. 14, 1950, received 200 mg. cortisone acetate daily.

TABLE IV. TESTS OF HEPATIC FUNCTION IN PATIENT WITH BILIARY CIRRHOSIS DURING CORTISONE THERAPY (SUBJECT S)

TIME (1950)	TOTAL PROTEIN (GM.)	ALBUMIN (GM.)	GLOBULIN (GM.)	BILIRUBIN (MG. PER 100 C.C. SERUM)		THYMOL TURBIDITY	$ZNSO_4$ TURBIDITY	CEPHALIN-CHOLESTEROL FLOCCULATION	ALKALINE PHOSPHATASE	PROTHROMBIN TIME (SEC.)
				DIRECT	INDIRECT					
1/21	6.5	3.7	2.8	3.6	1.0	8	17	Neg.		18
1/28	6.5	3.8	2.7							
1/30				4.5	1.1	10	13	Neg.	41.6	18
2/ 3*	6.3	3.6	2.7							
2/ 7				4.7	1.4	13	15	Neg.	47.6	18
2/ 9	6.3	3.7	2.6							
2/15	5.9	3.6	2.3	4.3	1.2					
2/17				4.6	1.8	6	11	Neg.	45.2	18
2/21	6.6	4.3	2.3	4.7	1.7					
2/27	6.9	4.4	2.5	5.6	1.0					
2/28				4.6	1.2	8	13	Neg.	43.6	18

*From Feb. 3, 1950, through Feb. 14, 1950, patient received 200 mg. cortisone acetate daily.

The blood fats were not altered (Table III) nor were the results of tests of hepatic function, serum bilirubin, or protein changed during administration of cortisone (Table IV). The elevated sedimentation rate remained high throughout the study (Table V).

TABLE V. LABORATORY DATA ON PATIENT WITH BILIARY CIRRHOSIS RECEIVING CORTISONE ACETATE (SUBJECT S)

TIME (1950)	HEMOGLOBIN (GM.)	ERYTHROCYTES*	LEUCOCYTES†	CELL VOLUME	SEDIMENTATION RATE (MM. PER HR.)
1/19	10.6	3.82	8.1		
1/30	10.0	3.72	7.4	36	
2/3‡					118
2/7	10.8	3.36	9.2	36	124
2/9					120
2/15					106
2/17	9.8	3.33	11.4		100
2/21					116
2/28	10.3	4.14	11.7	39	114

*Millions per cubic millimeter of blood.
†Thousands per cubic millimeter of blood.
‡From Feb. 3, 1950, through Feb. 14, 1950, patient received 200 mg. cortisone acetate daily.

METABOLIC DATA

Only Patient S was on a complete balance study.

Nitrogen Balance.—During control periods 1 and 2, nitrogen balance was positive to the extent of 0.55 and 0.04 Gm. per day respectively (Fig. 2). On initiation of administration of cortisone acetate (200 mg. per day), urinary excretion of nitrogen increased the first day and gradually thereafter. The average total output of nitrogen during the first six days on cortisone (period 3) was 13.82 Gm. per day, and during the second six days (period 4) it was 15.09 Gm. per day, representing increases, as compared with the immediately preceding control period, of 1.32 and 2.59 Gm. per day. Administration of cortisone was stopped at the beginning of period 5, but nitrogen balance remained negative. During period 6, however, urinary nitrogen excretion returned to the levels observed before cortisone was given, and the balance again became positive. Fecal nitrogen excretion did not vary greatly in any of the periods except 6, in which excretion appeared to be significantly reduced.

Creatine and Creatinine.—Urinary excretion of creatinine appears to have definitely declined in period 4 during the second six days of administration of cortisone (Fig. 3). In periods 5 and 6 creatinine output decreased still further, until the output in period 6 amounted to only 366 mg. of creatinine nitrogen per day, as compared with the average daily excretion of 435 mg. before cortisone treatment. Creatine excretion, which was equivalent to about 140 mg. of nitrogen per day before administration of cortisone, promptly decreased to less than 100 mg. per day during administration of cortisone but returned to the control level after withdrawal of cortisone.

Calcium and Phosphorus.—The average excretion of calcium in the urine during control periods 1 and 2 and the first six days of administration of cortisone (period 3) was 99, 111, and 105 mg. per day respectively (Fig. 2). During the second six days of administration of cortisone (period 4) and the subsequent control periods 5 and 6, urinary excretion of calcium increased significantly to 128, 186, and 171 mg. per day. At the same time fecal excretion of calcium

varied considerably, increasing rather markedly in period 3, but then decreasing again until the output in period 6, 1.06 Gm. per day, was not much more than half of that in period 3. Because of these large shifts in fecal excretion of calcium, the balance changed from strongly negative in period 3 to strongly positive in period 6. Urinary excretion of phosphorus remained remarkably constant throughout the period of observation and seemed to be more or less unrelated to urinary nitrogen, urinary calcium, or administration of cortisone. Fecal excretion of phosphorus increased somewhat in periods 4 and 5 (Fig. 2).

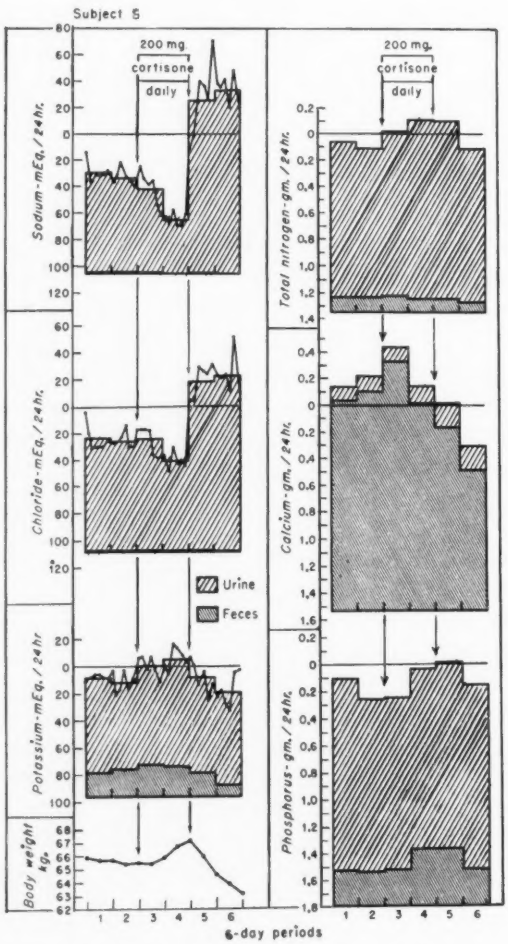

Fig. 2.—Metabolic data on Patient S.

Potassium, Sodium, and Chloride.—The initial effect of cortisone was to cause a significant increase in the urinary excretion of these substances. Urinary excretion of potassium remained elevated throughout the twelve days of administration of cortisone, the total output in excess of that in control period 2 being 142 meq. (Fig. 2). Fecal potassium also increased slightly during this

period, and potassium balance became negative. After administration of cortisone was discontinued, urinary excretion of potassium returned to approximately pretreatment level. Fecal potassium also decreased markedly, and potassium balance again became positive.

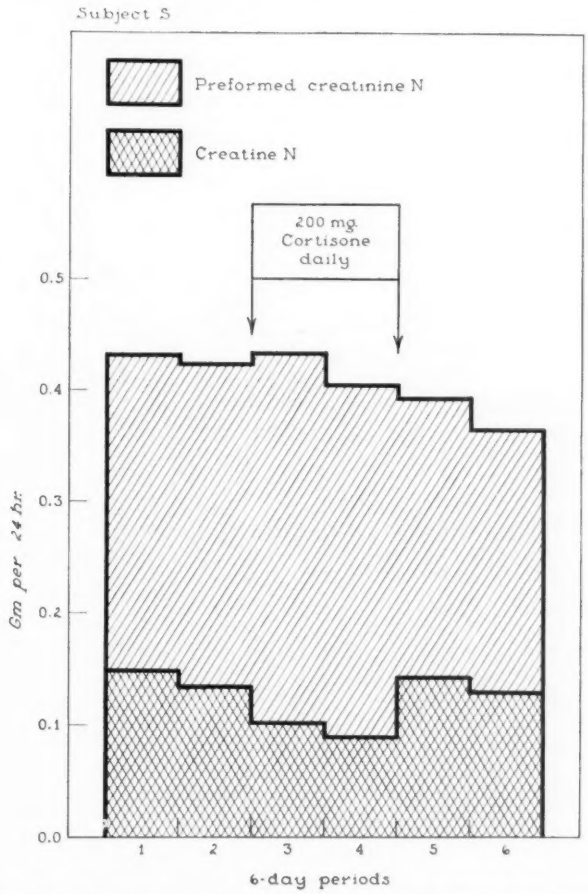

Fig. 3.—Urinary excretion of creatine and creatinine by Patient S.

The initial increase in excretion of sodium and chloride, which occurred when administration of cortisone was started, soon changed direction, and the balance for these electrolytes, already unaccountably positive in this patient during control periods 1 and 2, became even more strongly positive. Retention of sodium in periods 3 and 4, as compared with that in period 2, amounted to 245 meq., and of chloride, 80 milliequivalents. On cessation of administration of cortisone, excretion of sodium and chloride increased rapidly and enormously, and the strongly positive balances observed during administration of cortisone became strongly negative in periods 5 and 6. The total changes in balances in the latter two periods, as compared with the positive balances in period 4, represented a loss of 1,144 meq. of sodium and 748 meq. of chloride.

Plasma Electrolytes.—The concentration of sodium in the blood plasma decreased from 130 meq. per liter to 126 meq. per liter during the twelve days of control observations in periods 1 and 2, although sodium balance was positive during these periods (Fig. 4). During the twelve days of administration of

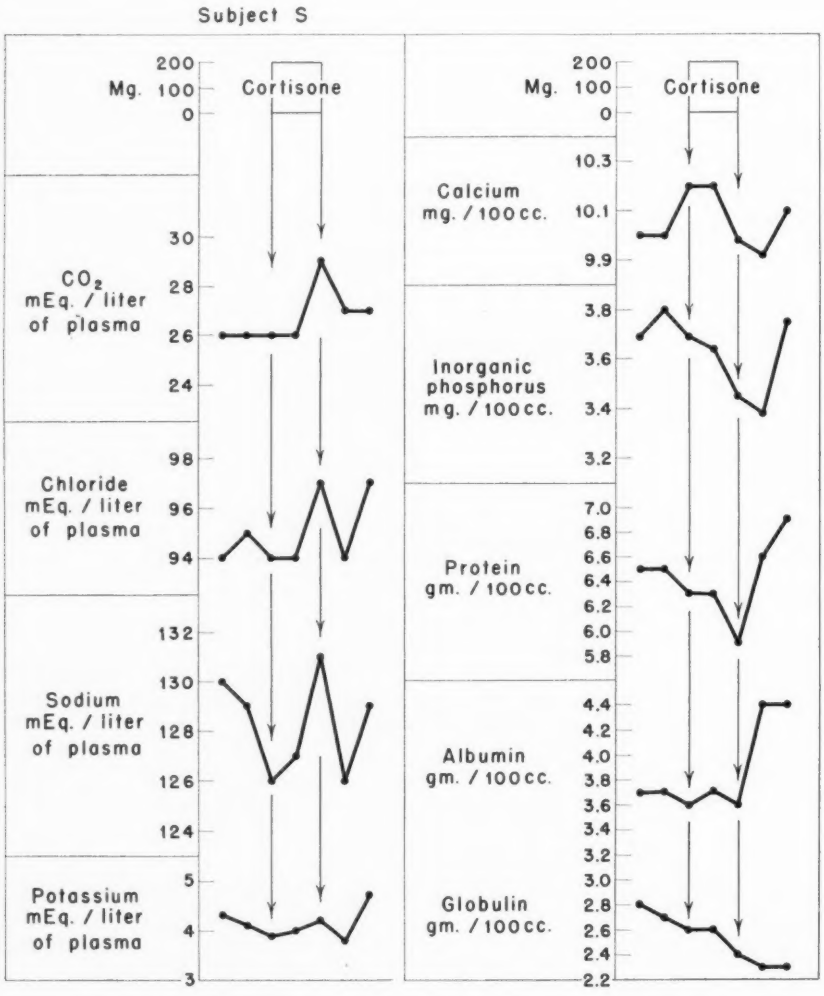

Fig. 4.—Concentrations of electrolytes in the plasma of Patient S.

cortisone, plasma sodium increased to 131 meq. per liter. Carbon dioxide content of plasma and chloride also increased during administration of cortisone. Serum potassium content did not change significantly nor did serum calcium content, which fluctuated within the narrow range of 9.9 to 10.2 mg. per 100 cubic centimeters. Plasma inorganic phosphorus values decreased somewhat during and after administration of cortisone. Serum protein concentration also decreased, apparently mainly in the globulin fraction. In view of the

large quantity of lipid material in the serum of this patient, some uncertainty may exist as to the actual value of the protein fractions.

Total Lipids, Cholesterol, and Bilirubin.—The data show that the values for these constituents fluctuated considerably before, during, and after administration of cortisone. An attempt to relate the changes to the effects of cortisone seems unwarranted (Fig. 5).

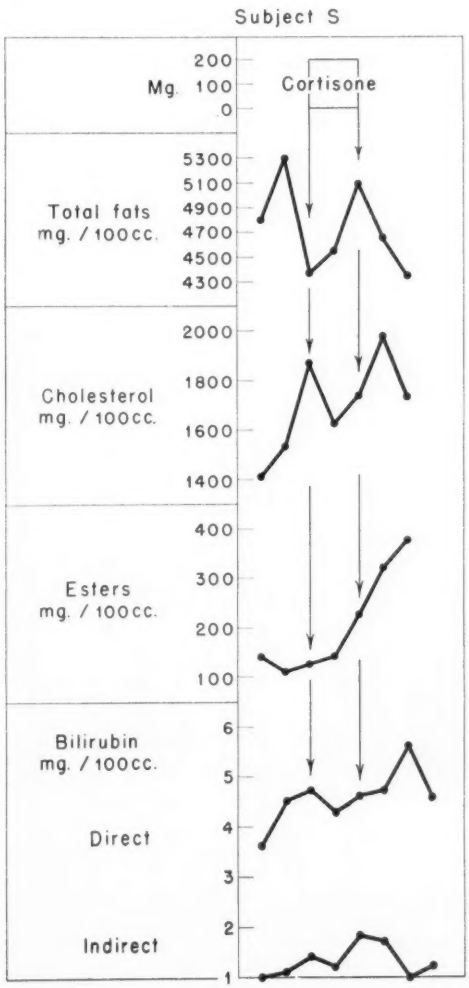

Fig. 5.—Total lipids, cholesterol, and bilirubin for Patient S.

HORMONE EXCRETION

In both cases the excretion of 17-ketosteroids before administration of cortisone was quite low. The average value for the woman (Case S) was 3.0 mg. per twenty-four hours with a range of 2.2 to 3.5, and corresponding values for the man were 2.4, and 1.0 to 4.9 mg. per twenty-four hours. When other patients with rheumatoid arthritis who excreted such small amounts of 17-ketosteroids

before treatment were given 200 mg. of cortisone acetate daily the amount of urinary 17-ketosteroids increased gradually to levels of 6 to 14 mg. per twenty-four hours. However, in these cases of hepatic disease the excretion of 17-ketosteroids was depressed even further. During the fourteen-day period the amount excreted by the male patient (Case G) gradually decreased to 0 on the last day of treatment (Fig. 6). During the second period of administration of 100 mg. of cortisone per day the excretion of 17-ketosteroids by the male patient was again depressed. The urine of the woman (Patient S) contained no 17-ketosteroids on the third and fourth days, but there was a small increase to an average value of 1.8 mg. during the remainder of the period of administration (Fig. 7). This average value is definitely less than the average of seven determinations before treatment.

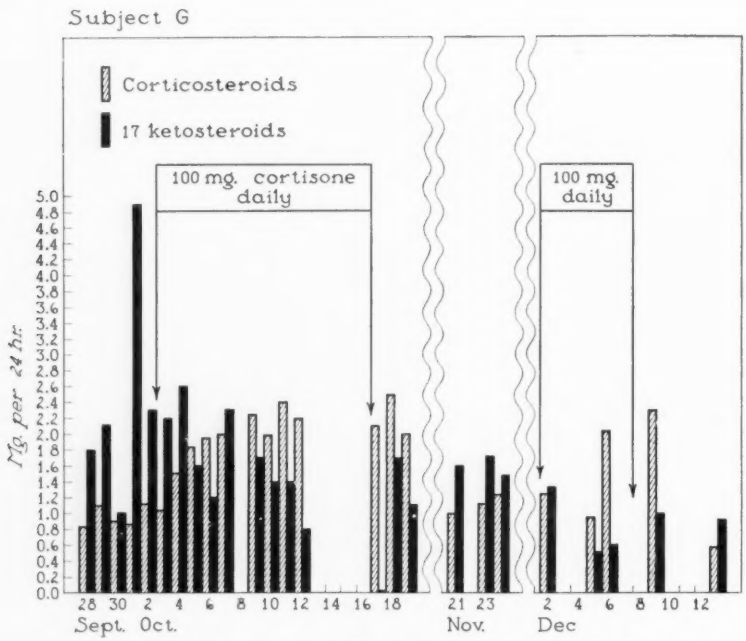

Fig. 6.—Excretion of corticosteroids and 17-ketosteroids by Patient G.

Patients with hepatic disease usually excrete very small amounts of 17-ketosteroids; the reason is not known. However, an obvious interpretation is that the damaged liver is unable to convert adrenal or testicular precursors into 17-ketosteroids as effectively as the normal liver. Also testicular function and some phase of adrenal function may be impaired in liver disease. There is evidence that administration of 100 mg. of cortisone acetate daily causes a suppression of the function of the recipient's adrenal cortex.[1] If that is true then the only source of 17-ketosteroids in the case of the woman would be the cortisone which was administered. Comparison of the effect on the urinary 17-ketosteroids in other patients with rheumatoid arthritis and in these patients with hepatic disease suggests that the damaged livers were incapable of convert-

ing cortisone to 17-ketosteroids to the same degree as a normal liver. The complete disappearance of 17-ketosteroids from the urine of the woman patient is believed to represent a suppression of endogenous production of 17-ketosteroids from an adrenal source. The later reappearance of 17-ketosteroids in the urine would then represent a small conversion of the administered cortisone to 17-ketosteroids.

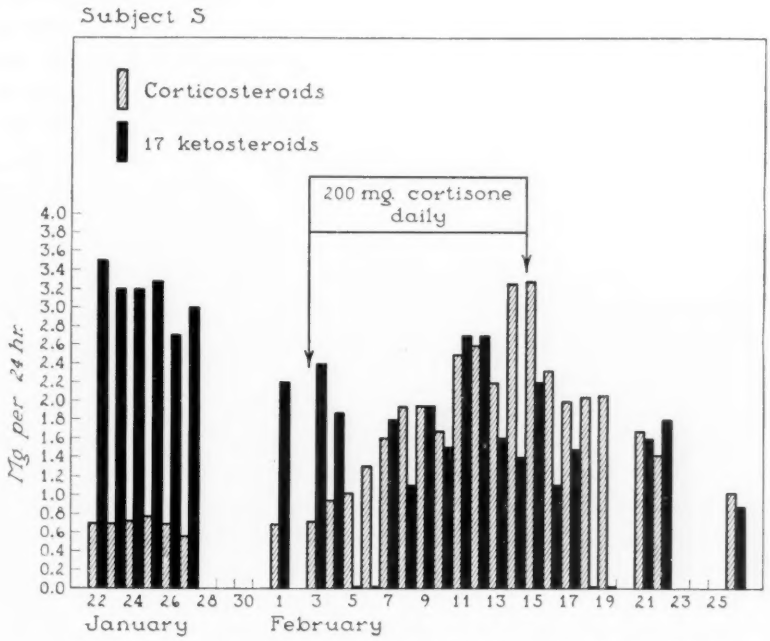

Fig. 7.—Excretion of corticosteroids and 17-ketosteroids by Patient S.

The amounts of corticosteroids excreted before treatment by both patients were within the range of normal values: the amounts excreted by the man (Patient G) were in the upper range of normal level. In both cases there was an increase in excretion of corticosteroids during the administration of cortisone acetate. The peak level obtained by the woman (Patient S) was about 3.3 mg., which may be compared with peak values of 5 to 6 mg. observed in three women patients with rheumatoid arthritis who received 200 mg. of cortisone acetate daily. If the liver is the site of inactivation and degradation of steroids one might have anticipated a smaller destruction of cortisone and therefore a greater excretion of corticosteroids in the urine. However, the amounts of corticosteroids excreted were probably not significantly different from those observed in cases of rheumatoid arthritis which was not accompanied by hepatic disease.

It may be concluded that no more than the usual amount of cortisone escapes metabolism when hepatic function is impaired but that under these circumstances less of it is converted to the 17-ketosteroids which appear in the urine.

DISCUSSION

Certainly in these two patients with hepatic disease the administration of cortisone acetate was not followed by any significant change in the results of tests of hepatic function or the blood lipid. The serum bilirubin changes were compatible with the natural history of the disease. The available literature on similar studies is meager and the reports are fragmentary.

Thorn and associates[2] have reported data on two cases in which jaundice was treated with ACTH. In one case of homologous serum jaundice superimposed on an aplastic anemia the patient was given a total of 1,060 mg. of ACTH over a period of eighteen days. A rapid loss of fever and marked increase in appetite were noted and in addition there was improvement in the results of hepatic function tests and a rapid fall in serum bilirubin. Observing the charted changes shown in this case one is impressed that similar changes have been noted in many patients with serum hepatitis who have not received any specific therapy. For this reason their assumption that ACTH might be responsible for such changes seems unwarranted. Their second patient had icterus following therapy with methyl testosterone. Again a marked increase in appetite and improved sense of well-being were noted but no sustained improvement in hepatic function was observed.

Sprague and associates[1] noted that the zinc sulfate turbidity reaction decreased significantly in twenty patients with arthritis during administration of cortisone and that the decrease was maintained after administration of the hormone was discontinued. We did not note any significant change in this reaction in these two patients. The same authors did not note any consistent pattern of change in the periodic determination of the serum alkaline phosphatase of four patients with arthritis following administration of cortisone or ACTH.

Bongiovanni and associates[3] gave seven patients with various types of cirrhosis of the liver 50 or 100 mg. daily of ACTH for periods of six to twenty-one days. Those patients with biliary cirrhosis and severe alcoholic cirrhosis with ascites showed no significant improvement. In four young women with cirrhosis of unknown origin the elevated gamma globulin fell and serum albumin rose following ACTH administration.

Adlersberg and associates[4] gave cortisone to ten patients with a variety of diseases in doses of 60 to 200 mg. a day for six to seventeen days. They noted usually a gradual elevation of serum phospholipids with a slower increase of serum cholesterol. Similar changes followed administration of ACTH.

Bluemle and associates[5] described one case of portal cirrhosis in which ACTH was given without effect. Their second patient with hepatic injury had a clinical response to ACTH which they described as being as striking as that seen in rheumatoid arthritis. The third patient with chronic hepatitis had a rapid fall in serum bilirubin following administration of ACTH.

The serum lipids and cutaneous lesions in the patient (Case S) with biliary cirrhosis in this study were essentially uninfluenced by the administration of 200 mg. of cortisone acetate daily. The collateral metabolic data on nitrogen

and electrolyte balances are presented primarily for the purpose of the record and for comparison with the responses of similar types of patients to cortisone. As to nitrogen, the increased excretion associated with a dose of cortisone acetate as large as 200 mg. per day was somewhat less than that observed by Sprague and associates[1] in patients with rheumatoid arthritis (compare especially cases 11 and 14). Creatine excretion (Case S) decreased during cortisone therapy, in contrast to the striking increases observed in cases 6, 11, and 14 of the paper previously mentioned. This difference invites speculation as to the extent to which damage to the liver may influence the synthesis of creatine. Whether creatine synthesis is depressed, possibly because of relative excess of androgen dependent on decreased hepatic inactivation of androgenic steroids, or whether some of the other steps in creatine synthesis not related to the effects of a steroid such as testosterone might be at fault in certain types of hepatic damage is questionable.

The effects of cortisone on electrolyte excretion in this case were similar to those seen in many other patients; namely, increased excretion of potassium with strong retention of sodium and chloride (after a short initial period in which excretion of sodium and chloride was increased). In the plasma, hypokalemic hypochloremic alkalosis, which occurs in some cases when cortisone is given at the 200 mg. level, failed to develop, although carbon dioxide content did increase somewhat.

The serum protein changes, with decrease of serum globulin, coincide with previous experience, although as mentioned earlier the uncertainties attending fractionation suggest caution in accepting these changes.

SUMMARY

The administration of cortisone acetate to a patient with severe serum hepatitis and to one with biliary cirrhosis with a marked hyperlipemia had no significant effect on the serum bilirubin, tests of hepatic function, the lipemia, the physical findings, or the course of the diseases.

Metabolic data on the patient with biliary cirrhosis were likewise not very significant, but are recorded for purposes of comparison with responses that may be noted by others in future studies of hepatic disease.

The excretion of 17-ketosteroids and corticosteroids in these two patients suggests that no more than the usual amount of cortisone escapes metabolism when hepatic function is impaired. However, with hepatic damage less of the administered cortisone is converted to the 17-ketosteroids which appear in the urine.

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PHOTOCHROMATOGRAPHIC ANALYSIS OF AMINO ACIDS IN BODY FLUIDS

A Method and Some Applications

Anthony A. Albanese, Ph.D., Reginald A. Higgons, M.D., Betty Vestal, B.S., and Loukia Stephanson
New York, N. Y.

PREVIOUS investigators have developed several variations of the single and two-dimensional techniques which are based on the "descending"[1] or "ascending"[2] principles. The procedures described in this report are based on the ascending principle which in our experience affords the maximum ease of operation. We have found that for most purposes the single dimensional technique provides a rapid means of obtaining satisfactory results. When the identification of unusual components is being attempted, the two-dimensional procedure should be employed.

ONE-DIMENSIONAL TECHNIQUE

Great simplification in design and operation of the partitioning chamber was secured by the use of the ascending rather than descending principle. The components of the apparatus are shown in Fig. 1,*A*. The outer structure of the chamber consists of a rectangular museum jar. This is made airtight as follows. A piece of wax paper is laid across the top of the museum jar. The ground surface of the edge of the cover is liberally coated with melted paraffin. Just before the paraffin solidifies, the cover is pressed firmly into place on the top of the jar. After a time sufficient for the paraffin to solidify thoroughly, the wax paper and the superimposed cover are both lifted from the jar. The wax paper is then gently peeled from the paraffin layer to leave an accurately fitting impression in the paraffin corresponding to the top of the jar. With careful handling such a jar cover can be applied and removed repeatedly for succeeding tests and still remain airtight.

The rack which supports the paper strips is constructed of strips of pine, 1 in. by ¾ in. joined together with Duco cement as shown in Fig. 1,*A*. Wire brads, 1½ in. long, nailed through the wooden frame form the suspension points. The frame is supported at the required distance from the bottom of the jar by 7 mm. Pyrex glass rods which pass through tightly fitting holes in the end sections of the wooden frame (Fig. 1,*A*). Two pony glasses placed diagonally opposite at the bottom of the jar complete the apparatus. The strips are dried without removing from the rack in the drying pan which was constructed as shown in Fig. 1,*B*.

The sampling table was assembled from sections of ¼ in. plate glass as shown in Fig. 1,*C*. The samples are measured with Sahli-type hemoglobinometer pipettes. It is advisable to have a supply which will measure 10, 20, and 25 c.mm., 20 and 30 c.mm., and 25 and 50 c.mm., so that all necessary aliquots can be measured.

The strips of paper, 1.8 by 29 cm., employed are cut from 58 by 58 cm. squares of Carl Schleicher and Schuell Co. #507 filter paper. This is best done by a stationer.

Procedure.—After many trials with various solvents, it had been found that pure phenol saturated with water provides the most convenient and reliable partitioning agent for the amino acids. This is made by adding 500 c.c. of water to a 1 lb. bottle of phenol crystals, Merck reagent grade, and allowing the mixture to stand about a week with intermittent

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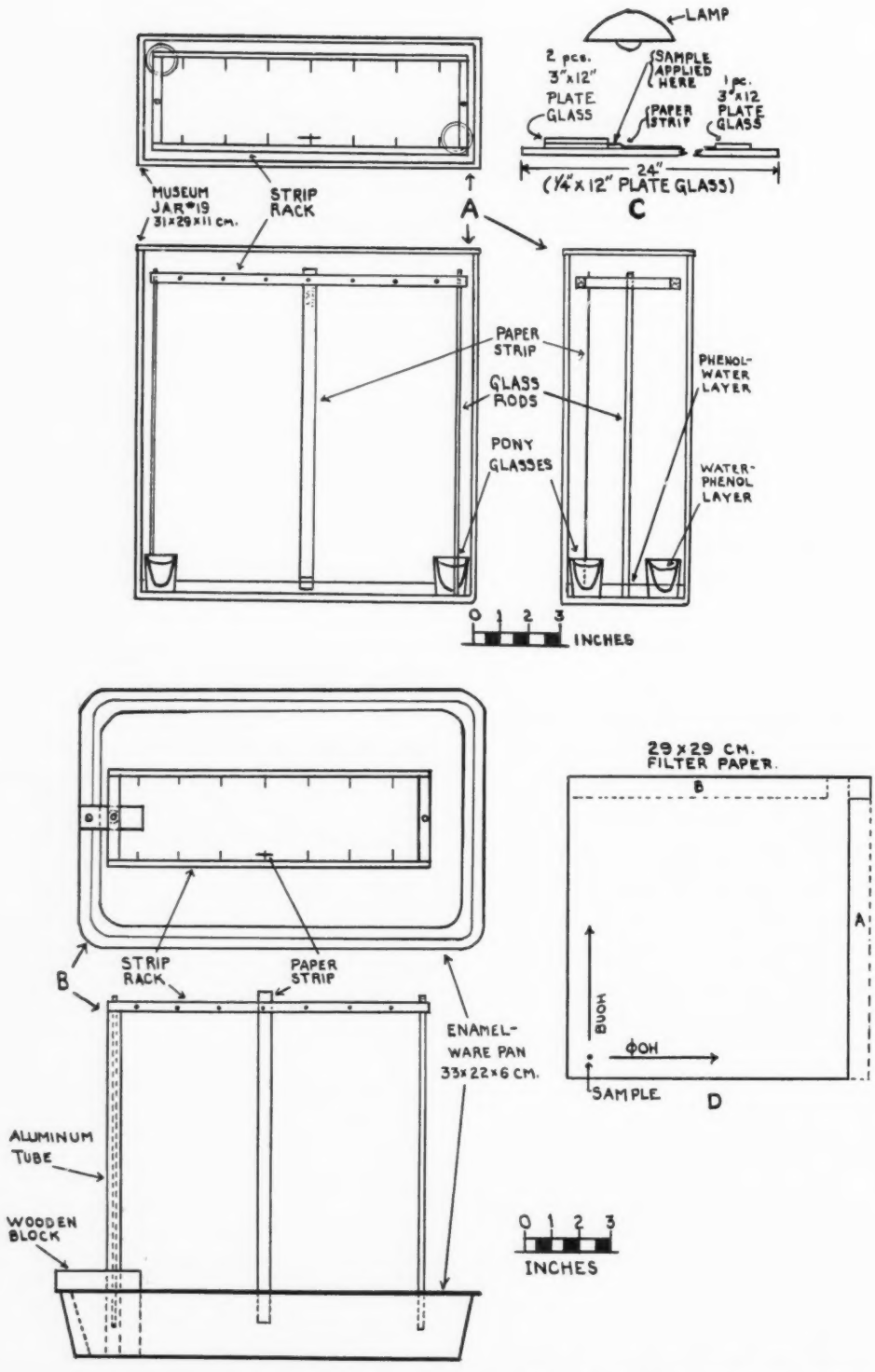

Fig. 1.——Drawing of apparatus for chromatographic analysis. *A*, Projection views of partitioning chamber and supporting rack for paper strips. *B*, Side and top view of drying pan with supporting rack in position. *C*, Side view of sampling table. *D*, Cut out details for filter paper to be used for two-dimensional analysis.

shaking before separation. Both layers, the phenol-water fraction and the water-phenol fraction, are collected in glass-stoppered bottles. The bottom of the museum jar is then covered with a layer about 1 cm. deep of the phenol-water fraction. The two pony glasses are filled within 1 cm. of the top with water-phenol fraction and set at opposite corners of the bottom of the jar. Before use, the partitioning chamber should be allowed to come to phase equilibrium which requires forty-eight to seventy-two hours. A well-sealed chamber can be used continuously, with occasional replenishing of the solutions, for at least twelve months.

In setting up an analysis, identifying notations are printed clearly within 2 cm. of the top and a line is drawn across the strips at 2 cm. from the bottom. The strips are placed on the sampling table and held in place by sandwiching between plate glass sections (Fig. 1,*C*). The samples are applied by drawing the pipette over the pencil line. Three or four applications are required for a 20 c.mm. aliquot. To avoid diffuse bands the sample should be applied in the thinnest possible line and the paper should be allowed to dry completely between applications. Drying can be considerably hastened by placing a lighted student's gooseneck lamp over the area (Fig. 1, *C*).

When the sampling process has been completed the strips are hung on the rack in the jar so that the sample line is about 1 cm. above the level of phenol-water layer. The jar is then covered with the paraffined glass top. The partitioning chamber should be placed in a draftless area, preferably an enclosed cabinet. With suitable samples and *temperature conditions* (22° to 25° C.) adequate separation is achieved in about eighteen to twenty hours. Thus, if the analyses are begun in the late afternoon the separation will be completed conveniently by 9 to 11 A.M. of the next day. The solvent should be allowed to rise about 22 cm. above the fluid layer; this may take less or more than the eighteen to twenty hours. If the column is allowed to rise beyond this distance the bands become too diffuse.

At the end of the run the rack laden with the strips is transferred to the drying pan located in an exhaust hood. The level attained by the solvent is marked by a pencil line and the strips are allowed to dry thoroughly. The strips are developed by painting (½ in. brush) with a 5 per cent solution of ninhydrin (Pfanstiehl) in normal butyl alcohol which has been prepared one hour before use. Complete color development is attained on standing about six to eight hours at room temperature.

Inasmuch as the color fades within a few days and interfering color arises from ninhydrin reagent itself, it has been found useful for record purposes to make photographic reproductions of the chromatograms immediately. This is done as follows. The strips are placed in parallel formation on the glass of a 9 in. by 12 in. printing frame. Then a piece of 8½ in. by 11 in. Scona Reflex semi-matte, type C paper is laid over the strips, emulsion side down, and the printing frame back is strapped into place. The exposure can be made to any light source. We have found that an x-ray viewer provides a constant and uniform light source. The printing frame is placed on the surface of the viewer and the light turned on for ten to fifteen seconds. The exposed paper is processed in the same solutions and manner employed for x-ray films. The bands on the photochromatograms so obtained will of course be color density reversals of the bands on the paper strips. Positive reproductions can be obtained by emulsion-to-emulsion contact printing. In our experience the negative copies are much more satisfactory and useful.

PREPARATION OF SAMPLES

Protein Hydrolysates.—Satisfactory photochromatograms of hydrolysates derived by acid or enzymatic hydrolysis can be obtained by the use of 10 or 20 c.mm. of solutions containing approximately 0.5 mg. of N per cubic centimeter. In practice when working with fractions of unknown composition it is advisable to make preliminary runs with varying amounts up to 50 cubic millimeters.

Urine and Ascitic Fluids.—In analyzing normal urine the size of sample will of course depend upon the total amino N content of the specimen which

can be determined readily by the copper method.[3] In our experience the urine sample must yield 4 to 5 γ of amino N per strip. Urines of infants contain 480 γ amino N per cubic centimeter (305 c.c. per day); those of older children 250 γ (1,050 c.c. per day); adults, 220 γ (1,640 c.c. per day); old adults, 170 γ (1,440 c.c. per day). Accordingly, the following amounts of urine should be employed for the various categories: infants, 10 c.mm.; older children, 20 c.mm.; adults, 20 c.mm.; old adults, 30 c.mm. On the basis of this information it is possible to set up the chromatographic analyses without an actual measurement of the amino N content of the urine. However, the analysis will be found to be more useful for interpreting metabolic developments and states in the subject if comparable amounts of amino N are employed in each determination.

In estimating the amino acid content of urines containing proteins, especially those from nephritic or nephrotic patients, the samples are prepared as follows. To 3 c.c. of urine in a 15 c.c. centrifuge tube are added with mixing 3 c.c. of acetone U.S.P. and after five minutes the conglomerate is centrifuged at 2,500 r.p.m. for ten minutes in a clinical centrifuge with an angle head. Depending on the condition of the patient, 10, 20, or 50 c.mm. may be required for a satisfactory analysis. Amino acid determinations of ascitic fluids are performed with samples prepared in the same manner as protein-containing urine Fifty cubic millimeter aliquots of supernatant fluid are needed.

Blood.—In determining the distribution of free amino acids in the blood, the samples are prepared as follows. To 0.5 c.c. of acetone in 15 c.c. centrifuge tube is added with vigorous shaking 0.5 c.c. of plasma, serum, or whole blood. After ten minutes the mixture is centrifuged and 50 c.mm. aliquots of the supernantant are applied to duplicate paper strips. The precipitated protein so obtained can be employed for total plasma or serum determinations. The remainder of the protein-free supernatant fluid can be used for other determinations, e.g. sodium[4] or potassium.

TWO-DIMENSIONAL TECHNIQUE

Except for differences in the partitioning chamber and other minor changes, the two-dimensional analyses are performed as described for the paper strip method. The apparatus consists of a pair of Pyrex glass cylindrical jars (6 in. by 18 in.) covered with 7 in. squares of ¼ in. plate glass. One of these contains a ½ in. layer of *n*-butyl alcohol saturated with water and a pony glass nearly filled with the water-butyl alcohol phase. Because of differential evaporation, the butyl alcohol-water layer must be replaced monthly. The other jar contains a ½ in. layer of phenol-water reagent with a pony glass nearly filled with water-phenol reagent.

The samples are prepared as previously described and double the amounts used for the one-dimensional strips applied at the lower left-hand corner (2 cm. from each edge) of 29 cm. squares which are obtained by quartering 58 by 58 cm. sheets (Fig. 1,*D*). A 2 cm. strip as indicated by the area A is cut out and a cylinder is formed by clipping the tab to the upper left-hand corner of the sheet. Care must be exercised that the two edges of the paper remain at least 1 cm. apart.

The cylinder is placed upright in the partitioning chamber containing the butyl-alcohol and water solvents. After seventy-two to seventy-eight hours the cylinder is removed from the jar and hung to dry in an exhaust hood. Drying can be extended over night or week end as suits the circumstances. The cylinder is now unfolded, the tab cut off, and a second one formed by cutting out area B (Fig. 1,*D*). A new cylinder is reformed by clipping the new

tab to the lower right-hand corner of the sheet. This is allowed to stand in the phenol-water chamber for about twenty hours. At the end of this time the phenol-soaked paper is hung in the hood and dried thoroughly.

The cylinder is then unrolled upon a piece of glass, 30 by 30 cm., painted with generous amounts of ninhydrin reagent and returned to dry in the hood. For purposes of permanent record the paper is cut 8½ in. by 11 in. and printed by direct contact with a piece of Scona Reflex paper of the same size.

INTERPRETATION OF RESULTS

Determination of Rf Values.—The Rf value is defined as the distance of movement of the solute divided by the distance of movement of the solvent measured in centimeters. The values given here were obtained by using 5 gamma quantities of the amino acids, (10 c.mm. of solutions containing 0.5 mg. of amino acid per cubic centimeter) of the individual amino acids: phenylalanine and isoleucine, 0.85 to 0.80; methionine, tryptophane and valine, 0.77 to 0.75; histidine, tyrosine, and alanine, 0.59 to 0.55; threonine and glycine, 0.47 to 0.41; serine, glutamic acid, and arginine, 0.34 to 0.30; cystine and lysine, 0.21; aspartic acid, 0.18. In practice, it will be found that the Rf values vary with concentration of total N content of the sample and distribution of amino acids. These variations always involve a shift of the Rf spectrum upward or downward as a whole group, never a change in spatial sequence of any individual amino acid. After some experience the correction for these shifts presents no serious problems. Hence any scale, such as is affixed to chromatograms to follow, must be regarded as a relative rather than absolute standard.

The positional characteristics of the amino acids in the two-dimensional chromatogram were determined by the use of 2 to 10 gamma quantities (Fig. 2). Overlapping of areas will occur if the amount of amino acid exceeds the usual concentrations.

APPLICATIONS TO METABOLIC PROBLEMS

Utilization of D-*Amino Acids.*—Previous studies have demonstrated that the utilization of D-amino acids by man cannot be completely evaluated by nitrogen balance determinations. This is particularly true for the unessential amino acids.[5]

While nonchromatographic chemical measurements of urinary metabolites of the D-amino acids can be employed successfully for quantitating their physiologic availability, an understanding of the mechanism of utilization requires the determination of the amino acids in blood, which, because of sample quantity requirements, can be determined most readily by chromatographic procedures. The results obtained with a number of the D-amino acids will be reported elsewhere.

Our reassessment of the utilization of D-methionine in the light of these techniques will serve as a typical illustration of the procedures employed and interpretation of results. In Fig. 3 are shown the photochromatograms of the blood and urine of a 12-year-old boy who was given supplements of 1.5 Gm. of DL- and L-methionine respectively in a 6 oz. glass of milk on consecutive days. On the first or control day he was given only the milk. Measurements were

made on twenty-four hour urine collections and blood specimens collected one hour after giving the test doses. It will be observed that the free methionine in the blood rose to comparable levels above the norm with the administration of DL- or L-methionine. Examination of the urine chromatogram discloses the presence of large quantities of methionine only in the urine collected during

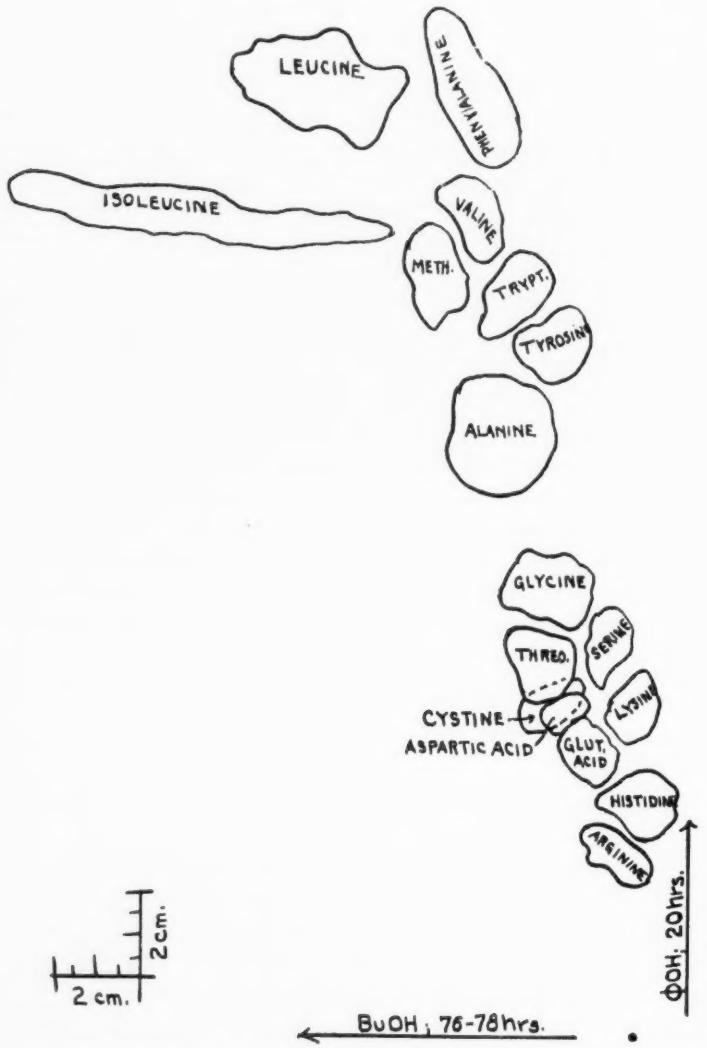

Fig. 2.—Spatial distribution of amino acids in two-dimensional separation.

the day on which DL-methionine was given. These findings suggest that the poorer utilization of DL-methionine is probably due to a lower kidney threshold for the D-portion of the racemate. This view is supported by parallel chemical analyses of the urine which disclose that whereas only 2 per cent of the L-methionine supplement is eliminated, 20 per cent of the D fraction of the DL-methionine

is lost by this route. Similar results were obtained when 3.0 Gm. of L- or DL-methionine were given as test doses. It is important to note that the ingestion of these amounts of L- or DL-methionine did not cause any appreciable shift in the free amino acid pattern of the urine or blood.

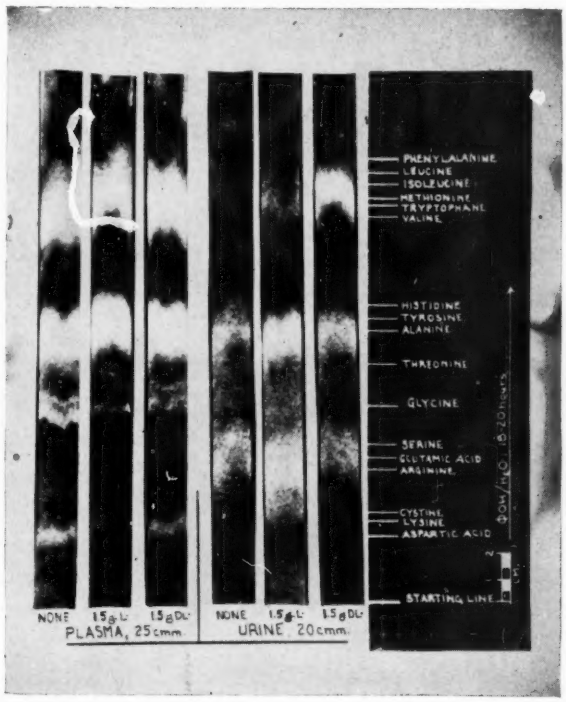

Fig. 3.—Photochromatographic amino acid pattern of urine and blood filtrate of a boy who received 1.5 Gm. of L- and DL-methionine.

Amino Acid Patterns of Body Fluids in Nephrosis.—A comparison of the blood, urine, and ascitic fluid chromatograms of a 5-year-old boy with nephrosis with the blood and urine chromatograms of a normal child of the same age yields some very interesting information (Fig. 4). It will be noted that there is no significant difference in the quantitative and qualitative distribution of amino acids in the urine of normal and nephrotic children. Examination of the plasma chromatograms, however, reveals marked qualitative and quantitative differences in free amino acid pattern. These are characterized by the absence of free aspartic acid, cystine, lysine, serine, arginine, and glutamic acid, and possibly isoleucine from the plasma of the nephrotic child which are usually present in the plasma of normal children. The presence of threonine, commonly absent in the plasma of normal children, is of interest because of the observations of Bauer and Berg[6] that mice maintained on threonine-deficient diets developed pronounced edema and ascites in twelve to twenty days. Attention is called to the similarity in amino acid pattern of the plasma and ascitic fluid of the nephrotic child. This is of special interest to us since an earlier study in this field by one of us disclosed that the proteins occurring in the ascitic fluid approxi-

mate in amino acid composition those found in the urine of the nephrotic subject rather than those found in normal plasma.[7] These observations constitute small but associated parts in a continuing study of the etiology and biochemistry of the nephrotic state.

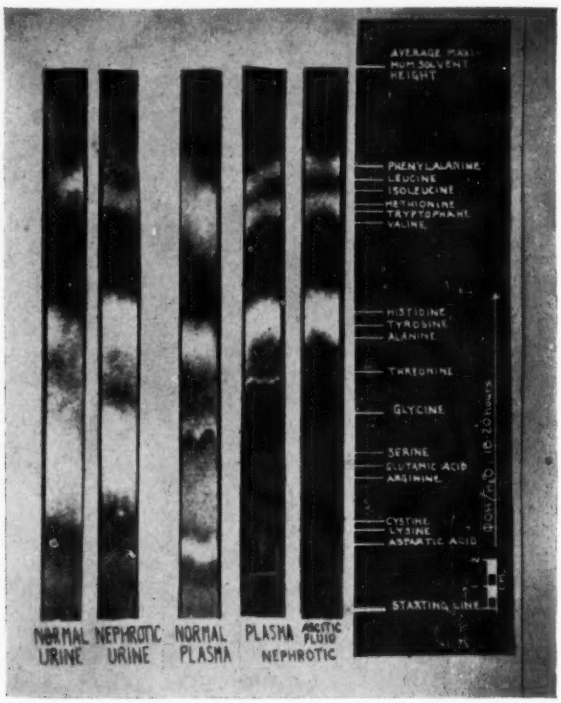

Fig. 4.—Photochromatograms of amino acids in body fluids of a normal and a nephrotic boy.

Other Applications.—Chromatographic analyses of urine of children with active or chronic rheumatic fever who received corticotropin (Wilson) or Cortone treatments showed an aminoaciduria having the same amino acid pattern as that determined microbiologically by Kemmerer and associates.[8] Our preliminary observations suggest that the aminoaciduria arises primarily from increased blood levels of the individual amino acids, in particular, histidine, lysine, and threonine.

In a previous report[9] the nitrogen-sparing effect of carbohydrates in man was shown to be achieved in part by a reduced urinary output of tryptophane and other unidentified amino acids. Application of photochromatography to the study of this mechanism has now disclosed that the reduction in amino acid excretion is paralleled by a uniform lowering of amino acid levels in the blood.

SUMMARY

A description of a modified apparatus and procedures for the photochromatographic analysis of amino acids in various body fluids has been presented.

The chromatographic solvents used are phenol-water and water-phenol. Ninhydrin in butyl alcohol is the chromatogenic reagent.

Application of the method to problems in metabolism in man includes (a) the utilization of D-amino acids, (b) amino acids in childhood nephrosis, (c) effect of adrenal cortical hormones on amino acids, and (d) the relationship of amino acids to the nitrogen-sparing action of glucose.

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QUALITATIVE AND QUANTITATIVE CHANGES IN THE PLASMA PROTEINS OF LIPOID NEPHROSIS DEMONSTRATED BY ELECTROPHORESIS

MARIE A. FISCHER, PH.D., PAUL A. STEINMAN, ANNA MARY CARPENTER, PH.D., AND MAUD L. MENTEN, M.D., PH.D., PITTSBURGH, PA.

THE continuous urinary loss of protein in children suffering with lipoid nephrosis is accompanied by quantitative changes in the protein components of the plasma. Although such changes may be indicated by chemical analysis, more detailed information is revealed by electrophoresis. In the present investigation, electrophoretic separation of the plasma proteins of seven children with lipoid nephrosis confirmed the previously published observations[1] that the percentages of albumin and gamma globulin are regularly lower; the fibrinogen, alpha$_2$ and beta globulin concentrations are higher; and the alpha$_1$ globulin concentration shows no consistent variation from that of normal adult plasma. It was apparent in our studies that the beta globulin area was composed of two well-resolved peaks. Particular attention was therefore given to this region in order to define qualitative differences between the beta globulins of normal and nephrotic plasma. Careful measurements of the mobilities of the plasma proteins were made and the effects of storage and dialysis on the electrophoretic patterns were observed. In addition, electrophoretic analyses were carried out in a barbital-caprylate buffer to further characterize the two peaks of the beta globulin area.

The use of caprylate in the buffer was suggested by Ballou, Boyer, and Luck,[2] who reported that a specific interaction between caprylate ion and human albumin caused an increase in the anodic mobility of the protein. Subsequently, Armstrong, Budka, and Morrison[3] found that in a barbital-caprylate buffer the beta globulin area of normal plasma was decreased and the alpha$_2$ globulin area correspondingly increased. Therefore, it seemed worth while to separate the proteins of nephrotic plasma and urine in a barbital-caprylate buffer and to determine whether the components of the pathologic samples were affected by caprylate anion.

EXPERIMENTAL

On the day of collection, 4 ml. of plasma were diluted with 8 ml. of buffer and dialyzed for two or three days at 2 to 4° C. against 2 liters of buffer, except in those experiements in which the effects of dialysis time and of caprylate ion were studied. In the latter studies, the plasma was dialyzed for one hour against 2 liters of buffer at room temperature according to the method of Fischer and Steinman.[4] Electrophoretic analyses were made immediately in some instances, in others only after a subsequent period of static dialysis at refrigerator temperature.

The two buffer solutions used were the standard 0.01N NaV-0.02N HV buffer at pH 8.6 and ionic strength 0.1, and the barbital-caprylate buffer system, 0.05N NaV-0.01N HV-0.05N

From the Departments of Physiological Chemistry and Pathology, University of Pittsburgh, School of Medicine, and the Pathological Laboratories of the Children's Hospital of Pittsburgh.

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Na-caprylate, at pH 8.6 and ionic strength 0.1. The electrophoretic analyses were carried out at 1.5° C. in an electric field of 8.0 volts per centimeter for 180 minutes. The patterns of the ascending boundaries were enlarged two and one-half times, traced, and the area of each component was determined by the method of Tiselius and Kabat.[5] The area of each component was compared to the total area of the pattern and was recorded as the percentage of the total area. The beta globulin disturbance made the descending patterns difficult to compare. Therefore, it was necessary to confine measurement of area to the ascending boundaries.

Mobilities of the components of both the ascending and descending boundaries were computed from the distance between each peak and the center of the boundary anomaly. The tabulated values also include the averages of the ascending and descending boundaries of each electrophoretic component.

RESULTS

The Protein Components of Nephrotic Plasma.—In most respects the electrophoretic patterns obtained in this study of plasma from children with nephrosis and from normal individuals were similar to those previously reported by Routh and co-workers.[1] However, resolution of the β-globulin area into distinct β_1 and β_2 peaks was so frequent that it seemed worth while to consider these globulins separately. Table I contains the data obtained from analyses of the first plasma sample collected from each of the seven children with nephrosis and from three normal adults. In all but one patient (E. F.), the beta globulin area separated into the $beta_1$ and $beta_2$ fractions. In this case, separation of beta from $alpha_2$ globulin was complete, but the $beta_1$ and $beta_2$ globulin fractions were indistinguishable. This is in contrast to one of the normal samples (L. N.), in which the $alpha_2$ and $beta_1$ globulin fractions had not separated, but in which their composite area was clearly separated from the $beta_2$ area.

TABLE I. PERCENTAGE COMPOSITION OF PLASMA PROTEIN COMPONENTS OF CHILDREN WITH NEPHROSIS

SUBJECT	PER CENT ALBUMIN	PER CENT GLOBULINS					
		α_1	α_2	β_1	β_2	ϕ	γ
Patient							
E. F.	5.7	2.9	29.3		47.1	13.2	1.7
J. S.	16.2	5.4	23.1	8.5	35.4	8.5	3.1
L. D. S.	26.0	8.1	17.9	12.2	17.9	11.4	4.9
R. D.	17.1	6.5	19.5	16.3	24.2	10.6	5.7
Y. E.	5.2	7.2	27.8	18.7	25.2	12.1	3.9
L. S.	8.0	5.5	24.1	8.0	35.9	17.3	1.3
R. S.	18.0	3.5	27.8	8.4	25.4	15.5	1.4
Normal							
E. R.	52.2	5.4	4.8	8.3	12.0	7.8	9.6
L. N.	54.3	3.8		11.4	11.4	6.2	12.9
N. G.	57.0	5.3	5.7	7.5	11.0	4.4	9.2

The mobility values upon which identification of the plasma protein components were made are summarized in Table II. The values for the descending pattern of normal plasma are in agreement with those obtained by others[6, 7] who have also calculated mobilities from the distance which each peak had separated from the boundary anomaly. The descending mobilities of the protein fractions of nephrotic plasma, except for the $beta_1$ and gamma globulin, seem to be slightly higher than normal, and the ascending mobilities are lower than

normal, with the exception of the beta$_2$ fraction. When, however, the ascending and descending mobilities of each boundary are averaged and the average mobilities of the normal and the nephrotic components are compared, the mobilities

TABLE II. MOBILITIES* OF PLASMA PROTEIN COMPONENTS OF NORMAL AND NEPHROTIC PLASMA

	PATTERN	NUMBER OF SAMPLES	ALBUMIN	GLOBULINS					
				α_1	α_2	β_1	β_2	ϕ	γ
Normal	Asc.	14	7.15	6.14	5.34	4.90	3.74	2.96	1.93
			±.11	±.12	±.08	±.13	±.15	±.02	±.11
Nephrosis	Asc.	27	7.00	5.94	5.03	4.61	3.85	2.83	1.88
			±.11	±.16	±.12	±.18	±.20	±.10	±.24
Normal	Desc.	14	6.48	5.38	4.47	4.00	3.18	2.26	1.56
			±.13	±.14	±.11	±.13	±.16	±.11	±.10
Nephrosis	Desc.	16†	6.61	5.54	4.53	4.04	3.33	2.39	1.55
			±.14	±.13	±.14	±.17	±.13	±.08	±.15
Normal	Average asc. and desc.		6.82	5.76	4.91	4.45	3.46	2.61	1.75
Nephrosis	Average asc. and desc.		6.81	5.74	4.78	4.33	3.59	2.61	1.72

*Average mobilities ($\times 10^5$cm.^{2} volt^{-1} sec.$^{-1}$) for each group of samples are given with the standard deviation for each mobility value.

†Descending patterns were not photographed on eleven of the analyses of nephrotic plasma.

of albumin, alpha$_1$ globulin, gamma globulin, and fibrinogen are practically identical. Slight differences are apparent, however, between the alpha$_2$, beta$_1$, and beta$_2$ globulin mobilities of normal and nephrotic plasma. In nephrosis, these globulin areas may contain either abnormal protein components or may represent increases in one specific component of the normal nonhomogeneous peaks. In the case of the beta$_2$ globulin component, evidence will be presented in this paper to show that the second explanation is probably the correct one.

The Effect of Prolonged Dialysis on Plasma Proteins.—Because the double beta-globulin peak observed in nephrotic plasma had not been pointed out previously, the question arose as to whether in other laboratories, and occasionally in our own, the slower of the beta globulins had undergone a mobility shift prior to analysis, so that it no longer migrated as a separate, readily defined peak. For this reason, the effects of dialysis time and storage on the nephrotic samples were investigated. One serum sample was divided into three portions. The first portion was dialyzed for two days and the second for seven days, while the third was refrigerated for five days prior to dialysis for two days. The patterns of Fig. 1, *a*, show that prolonged refrigeration caused an increase in the anodic mobility of beta globulin, but that the increase was more pronounced when the serum was dialyzed against the buffer for a comparable time. In both instances, the major portion of the beta-globulin had migrated more rapidly, but there was in addition a residual beta globulin peak of lower mobility. The changes due to prolonged dialysis are shown more strikingly in Fig. 1, *b* and *c*. In both cases, as soon as possible after collection, an aliquot of the plasma was dialyzed for one hour at room temperature and immediately an-

alyzed electrophoretically. A second portion of the sample was first rapidly dialyzed and then allowed to dialyze statically in the refrigerator for three or four days prior to electrophoresis. The aliquots which were rapidly dialyzed

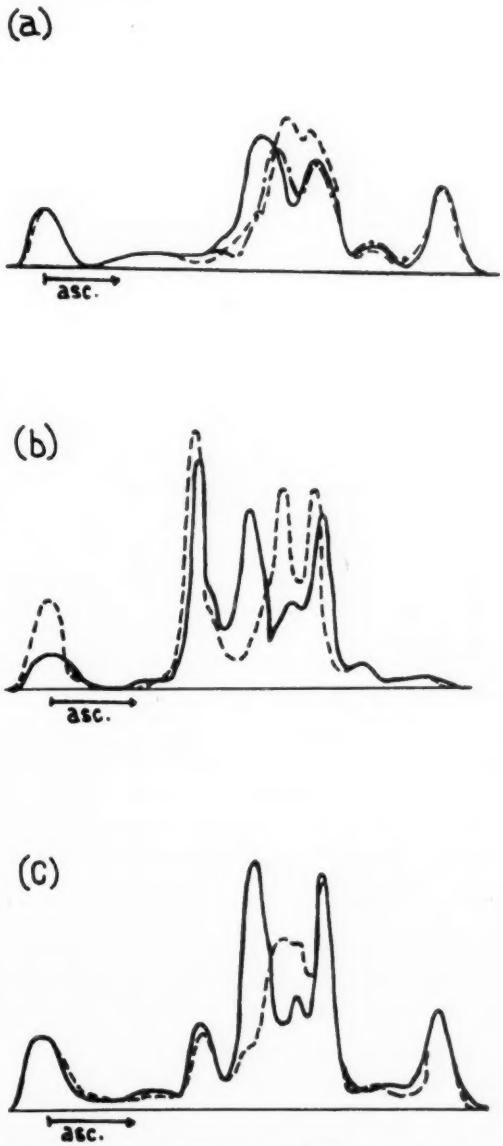

Fig. 1.—The effect of storage and prolonged dialysis on the electrophoretic patterns of nephrotic plasma or serum in the barbital buffer, pH 8.6.

a, Serum from Patient L. M. was analyzed after two days of static dialysis (————), and after five days of refrigeration followed by a two-day static dialysis (- - - - - -), and also after static dialysis for seven days (----------).

b, Plasma from Patient E. F. was analyzed after rapid dialysis for one hour (————) and also after rapid dialysis for one hour followed by static dialysis for three days (----------).

c, Plasma from Patient R. D. was analyzed after rapid dialysis for one hour (————) and also after rapid dialysis followed by four days of static dialysis (----------).

and then analyzed on the day of collection had distinct alpha$_2$, beta$_1$, and beta$_2$ globulin peaks. On the other hand, the beta$_2$ globulin of each aliquot which had been exhaustively dialyzed had an increased anodic mobility, and for this reason the beta$_1$ and beta$_2$ globulins no longer appeared as distinct electrophoretic components. It is evident from Fig. 1, *b* and *c*, that the major portion of the beta$_2$ globulin fraction of nephrotic plasma is composed of the beta$_2$ globulin which is sensitive to dialysis and that further electrophoretic studies of the beta globulin components of nephrotic plasma in the barbital buffer should be made only on samples which have been dialyzed rapidly and analyzed soon after collection.

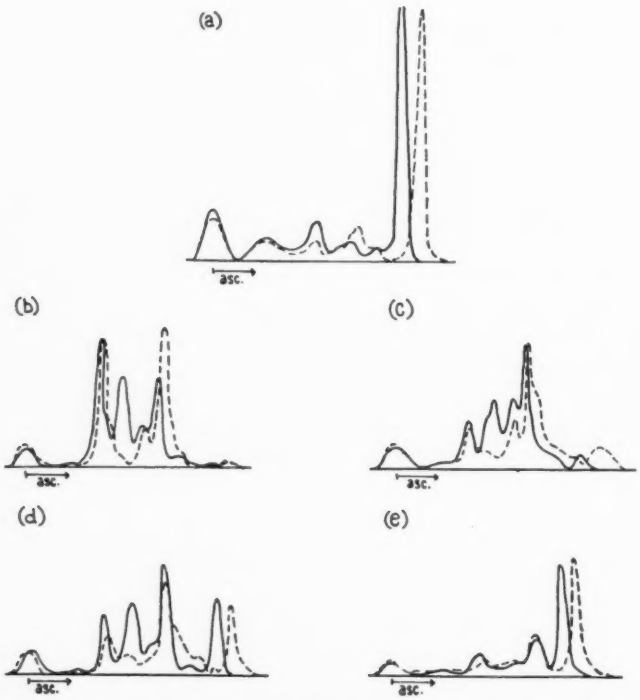

Fig. 2.—A comparison of the patterns obtained from electrophoresis in both the barbital buffer at pH 8.6 (————) and in the barbital-caprylate buffer at pH 8.6 (----------) of *a*, normal serum from Subject L. N., *b*, nephrotic plasma from Patient E. F., *c*, nephrotic plasma from Patient Y. E., *d*, nephrotic plasma from Patient R. S., and *e*, urine from Patient Y. E.

The Effect of Caprylate Ion on Blood Proteins.—(a) *In Nephrosis:* Use of the barbital-caprylate buffer at pH 8.6 seemed to offer a method whereby the protein components of nephrotic samples might be further characterized. Although Armstrong, Budka, and Morrison[3] found that caprylate effected no change in albumin mobility, in every instance studied in this laboratory the increase in albumin mobility was observed. No reason was apparent for this discrepancy, but the patterns of Fig. 2, in which each sample was analyzed in both the standard barbital and the barbital-caprylate buffer at the same pH, ionic strength, and protein concentration, show that the albumin of normal

serum (Fig. 2, *a*) and of nephrotic plasma (Fig. 2, *b*, *c*, and *d*) and urine (Fig. 2, *e*) has the common property of interaction with caprylate ion and thus has an increased anodic mobility in the barbital-caprylate buffer. Nephrotic as well as normal blood also contained the $beta_2$ globulin component which had an increased mobility and, therefore, appeared in the $alpha_2$ globulin region in the presence of caprylate. In normal serum, this component represented approximately one-half of the total $beta_2$ area found in the standard barbital buffer, but in the samples from nephrotic patients the major portion of the plasma $beta_2$ globulin region was composed of the caprylate-sensitive component. On the other hand, the beta globulin of nephrotic urine (Fig. 2, *e*) was not influenced appreciably by the presence of caprylate anion. In this respect, it differed from the major beta component found in the plasma of the same patient (Fig. 2, *d*).

(b) *In Other Abnormal Sera:** Even though reaction with caprylate in the normal plasma and in that of nephrotic patients seemed to be specific for albumin and one of the $beta_2$ globulin components, it was deemed worth while to study the effect of this ion on other abnormal sera which had an increased globulin concentration, in order to determine whether the elevated globulins contained a caprylate-sensitive fraction. Therefore, sera obtained from patients with multiple myeloma, lymphogranuloma venereum, and ulcerative colitis were analyzed both in the barbital and in the barbital-caprylate buffers. The increased mobility of the albumin fraction in the caprylate buffer, as well as the increased mobility of a $beta_2$ globulin component is again apparent. As was the case in normal samples, only one-half or less of the $beta_2$ globulin peak was composed of the caprylate-sensitive component. The globulin of multiple myeloma migrated with a mobility intermediate between the beta and gamma globulins and its mobility was not affected by caprylate. Furthermore, the increased gamma globulin components in the sera of patients with lymphogranuloma venereum and ulcerative colitis were also unaffected by the caprylate anion. These observations tend to confirm the fact that reaction with caprylate is a specific property of the albumin fraction and a $beta_2$ globulin fraction of human plasma.

DISCUSSION

An increased beta globulin content of plasma is one of the characteristic findings in nephrosis.[8] The present studies demonstrate that this beta globulin component is composed of at least two fractions and that the $beta_2$ globulin is present in larger quantities than the $beta_1$ globulin. Almost the whole of the $beta_2$ globulin fraction in nephrosis is markedly affected by storage in the refrigerator and by prolonged dialysis in the standard barbital buffer. Under these circumstances the mobility of the $beta_2$ component increases until the area of this fraction merges with that of the $beta_1$ globulin area. Since it is well known[3] that certain of the lipid-containing globulins are sensitive to storage and environment, it is probable that this $beta_2$ component is the same protein which has been ether extracted from nephrotic serum by Longsworth and MacInnes.[9]

*The authors are indebted to Dr. T. S. Danowski and Dr. Fred R. Franke for the samples of plasma from these patients.

Essentially all of the $beta_2$ globulin fraction of nephrotic plasma reacts with the caprylate anion, whereas only about one-half of the $beta_2$ globulin of normal plasma is affected. The caprylate reaction seems to be specific for one of the plasma $beta_2$ globulins and for albumin. One can speculate, therefore, that in nephrosis the increase in this particular $beta_2$ globulin is in response to the decreased plasma albumin concentration in order to sustain a function which is normally assumed by the albumin molecule. This particular role for the albumin molecule and for the $beta_2$ globulin of nephrosis may be elucidated when further studies explain the mechanism of the reaction between caprylate ion and albumin.

In another respect, however, the plasma $beta_2$ globulin of nephrosis is unlike albumin because it is not excreted as such in the urine. The present study has shown that the beta globulin of nephrotic urine is not affected by caprylate ion, but that plasma and urinary albumin in nephrosis were qualitatively similar in that both interacted with caprylate. This observation is in accord with the common experience that the protein components found in high concentration in the plasma of nephrotic patients do not appear in significant quantities in the urine. This generality is true of fibrinogen and $alpha_2$ globulin as well as the $beta_2$ globulin fraction of nephrotic plasma.

SUMMARY

In this study it has been shown that the beta globulin area of nephrotic plasma is composed of two distinct peaks. The mobility of the slower peak is increased by lengthening the time of storage and dialysis. As a result, separation of the beta peaks may not be observed if the time of preparation for electrophoresis is prolonged.

The average of the ascending and descending mobilities of the albumin, $alpha_1$ globulin, fibrinogen, and gamma globulin fractions of normal and nephrotic plasma are in good agreement. The averages for the $alpha_2$, $beta_1$, and $beta_2$ globulin components show slight differences.

The albumin fractions of normal serum and of nephrotic plasma and urine are alike in their ability to interact with caprylate anion. Only about one-half of the $beta_2$ globulin of normal serum is sensitive to this ion, but the greater part of the $beta_2$ globulin of nephrotic plasma and essentially none of the beta globulin of nephrotic urine are reactive.

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EFFECTS OF POTASSIUM CHLORIDE ON THE INTESTINAL MOTILITY IN HUMAN BEINGS AND DOGS

P. C. Gazes, M.D., J. A. Richardson, Ph.D., and M. deV. Cotten, B.S.
Charleston, S. C.

DURING a study of patients with potassium depletion secondary to intestinal obstruction, both mechanical and paralytic,[1, 2] it was noted that, in many instances, the intravenous administration of potassium chloride was associated with an increase in intestinal motility as judged by stethoscopic examination and the passing of flatus. These effects were most dramatic in cases with postoperative secondary paralytic ileus. In addition to vomiting, the body potassium was further depleted by the administration of excessive parenteral fluids such as saline and glucose. In view of the apparent beneficial effects of potassium chloride the presently reported experimental study was conducted to confirm these clinical observations.

Relatively few studies have been conducted concerning the role of potassium in intestinal motility. Most of these have been confined to observations of the action of this ion on the excised, normal intestine. Alvarez[3] found that potassium increased the tone and depressed the rate of intestinal contractions in isolated strips of bowel. Whitehead,[4] studying the responses of excised rabbit intestine to alterations of electrolytic concentrations of sodium, calcium, and potassium, found that potassium deprivation resulted in inhibition and a subsequent tendency to spasticity when this ion was added. In 1933, Ochsner and associates,[5] using dogs with high intestinal obstructions, observed that hypertonic salt solutions stimulated the adynamic gut; the addition of calcium and potassium solutions resulted in more marked effects.

METHOD

Thiry-Vella fistula operations were performed on nine mongrel dogs, of about the same weight, at a level approximately three feet above the ileocecal junction. After an interval of about ten days postoperatively, kymographic recordings of intestinal motility were made by means of a water-filled balloon inserted in the lumen of the fistula. The balloon was connected to a recording water manometer with approximately 30 cm. water pressure according to the method described by Jackson.[6] After the dog was accustomed to the procedure control recordings were made. With seven studies in six unobstructed dogs, following a previous control period, isotonic potassium chloride solution (1.14 per cent) was administered by intravenous infusion at the rate of 5 to 10 c.c. per minute. In three additional dogs, isotonic potassium chloride solutions were given only after the bowel had been obstructed. Electrocardiographic tracings were made at frequent intervals during all of these experiments alternating Lead L_2 with precordial Lead V_3 using a direct-writing EPL Cardiotron. When sinoauricular block occurred, the potassium infusion was discontinued. High intestinal obstruction was produced several days later by sectioning the bowel a few inches distal to the ligament of Treitz; cut ends were inverted and sutured.

From the Department of Pharmacology and the Department of Medicine, Medical College of South Carolina.

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Frequent recordings of intestinal motility were made following obstruction. Food and water were given ad libitum. Within three to fifteen days all of the animals developed generalized muscular weakness, dehydration, and lethargy characteristic of the clinical picture of hypopotassemia; four dogs died after obstruction prior to completion of study. When it became evident from the kymographic tracings that intestinal activity was markedly depressed, isotonic potassium chloride was administered intravenously by infusion as was done with unobstructed dogs. A total of thirty-one studies were conducted on nine animals; of this number, potassium chloride was administered on fourteen separate occasions.

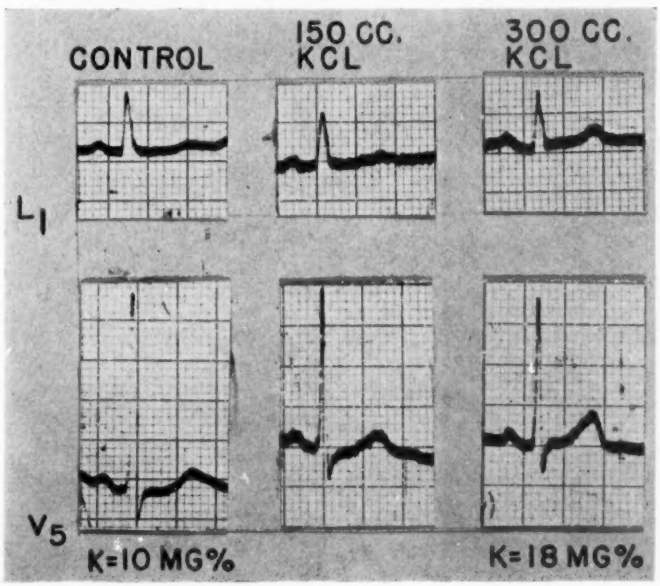

Fig. 1.—Electrocardiographic tracings of Leads L_1 and V_5 from a patient with adynamic ileus secondary to large bowel obstruction before and after the intravenous administration of 300 c.c. of isotonic potassium chloride solution. Serum potassium levels as indicated.

All observations on the unobstructed animals were preceded by a control period consisting of regular peristaltic contractions with small rhythmic movements in the intervals between the peristaltic waves. Such a preliminary control demonstrates a definite state of functional activity which serves as a relatively uniform basis for measurement of drug effects.

In the obstructed animals, potassium chloride was administered only after controls of one hour or longer had shown that there was relatively marked depression of intestinal motility.

RESULTS

1. *Clinical.*—Fig. 1 demonstrates the electrocardiographic changes in a patient with paralytic ileus, secondary to large bowel obstruction, before and after the administration of potassium. This 55-year-old man had been vomiting for eight days prior to admission and was weak, dehydrated, and, by stethoscopic examination, peristalsis was absent. In the control tracing, the T waves in Lead L_1 and precordial Lead V_5 are of diminished amplitude with slight S-T depression and prolongation of the Q-T interval to 0.5 seconds. The serum potassium was 10 mg. per cent. Following the administration of 300 c.c. of isotonic potassium chloride, the T waves became upright and the Q-T inter-

Fig. 2*A*.—Kymographic tracings of normal intestinal motility recorded from a Thiry-Vella fistula in an unanesthetized dog. Upper row of figures corresponds in time to electrocardiographic tracings shown in Fig. 2B. Three hundred and fifty cubic centimeters of isotonic potassium chloride solution were administered over a period of thirty-five minutes. Time marked in five-minute intervals.

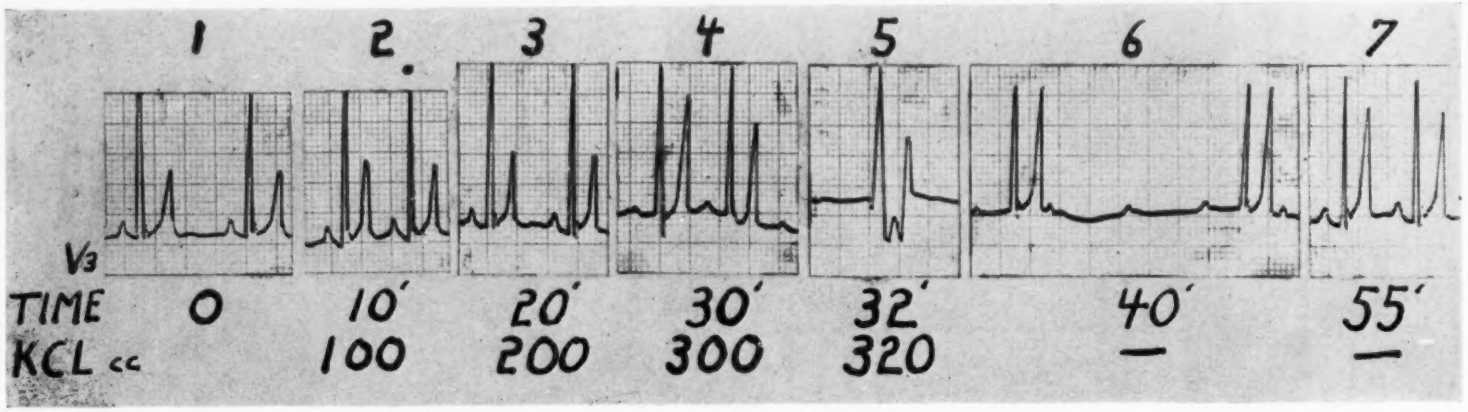

Fig. 2*B*.—Concomitant electrocardiographic tracings taken in the experiment shown in Fig. 2*A*. Upper row of figures corresponds to the numerals on the kymographic tracing.

val shortened to 0.3 second. The serum potassium level rose to 18 mg. per cent and the patient became less lethargic. At the same time, the patient began to pass flatus and peristaltic movements were audible on stethoscopic examination. These findings were also noted in nine other similar cases.

2. *Experimental.*—Fig. 2A illustrates the typical intestinal motility as recorded by the Thiry-Vella fistula preparation. The administration of 330 c.c. of isotonic potassium chloride over a period of thirty-five minutes produced no appreciable effect on the intestinal motility as measured by this "pressure-volume" method. The electrocardiographic changes which occurred during this administration of potassium chloride are demonstrated in Fig. 2B. Peaking of the T waves with shortening of the Q-T interval was the earliest manifestation. After the infusion of 300 c.c. of potassium chloride, the T wave was almost as high as the R wave. Two minutes later sinoauricular block occurred and shortly thereafter the infusion was discontinued. Twenty minutes after completion of the infusion, the electrocardiogram returned to control; this was preceded by an interval of second degree A-V heart block. Similar results were obtained in six other experiments.

Fig. 3A demonstrates the effects on intestinal motility of the administration of potassium chloride in an animal with high intestinal obstruction. It will be noted that in the control the intestinal motility was markedly depressed. The animal manifested typical signs of hypopotassemia of such a degree that it was unable to walk. After a control of one hour, an intravenous infusion of potassium chloride was begun. Following the administration of 50 c.c., intestinal motility increased markedly both with respect to rhythmical and peristaltic movements. In addition, the animal defecated, further indicating active intestinal motility. Following the administration of 160 c.c., the intestinal motility was comparable to that recorded in the normal dog. A total of about 225 c.c. was infused over a fifty-four minute period. Fifteen minutes following completion of the infusion the intestinal motility was still active. The animal appeared to be more alert and was able to walk without difficulty. In two other experiments, similar intensities of response were noted. In four additional experiments, in which there was less impairment of motor function, the stimulating action of the potassium chloride infusion was not as marked. Thus, in instances in which intestinal motility was active following obstruction, the effects of potassium chloride infusions on the bowel were not manifest; in instances in which there was a marked depression of the intestinal motility, potassium chloride infusions produced distinct stimulant effects. The inhibition of intestinal motility occurred as a rather late feature in these acutely obstructed dogs. Commonly ten days or more were necessary for development of an adynamic bowel.

Fig. 3B illustrates the electrocardiographic changes in the experiment shown in Fig. 3A. The control tracing of precordial Lead V_2 reveals S-T depression and inversion of the T wave with prolongation of the Q-T interval. Such changes have been described as being typical of hypopotassemia.[7] With the administration of potassium chloride, the S-T segment remained unchanged

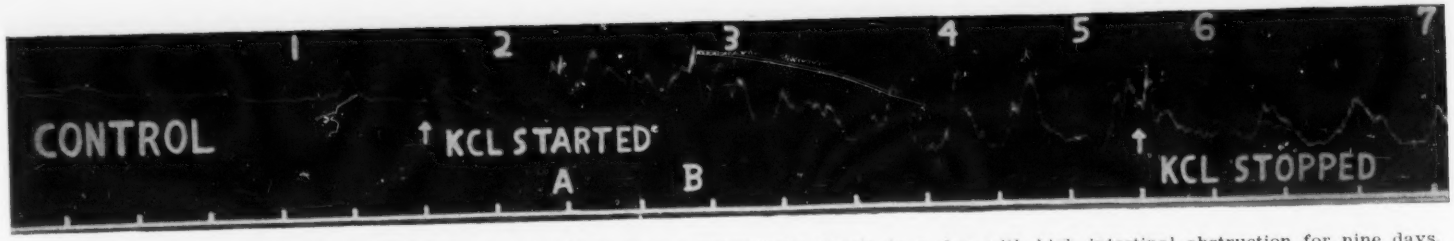

Fig. 3*A*.—Kymographic tracing of the intestinal motility from a Thiry-Vella fistula in a dog with high intestinal obstruction for nine days. A control of one hour preceded the administration of 224 c.c. of isotonic potassium chloride solution intravenously over a fifty-minute period. Letters *A* and *B* indicate the points at which defecation and vomiting occurred. Upper row of figures on the tracing refers to the electrocardiographic tracings shown in Fig. 3*B*. Time marked in five-minute intervals.

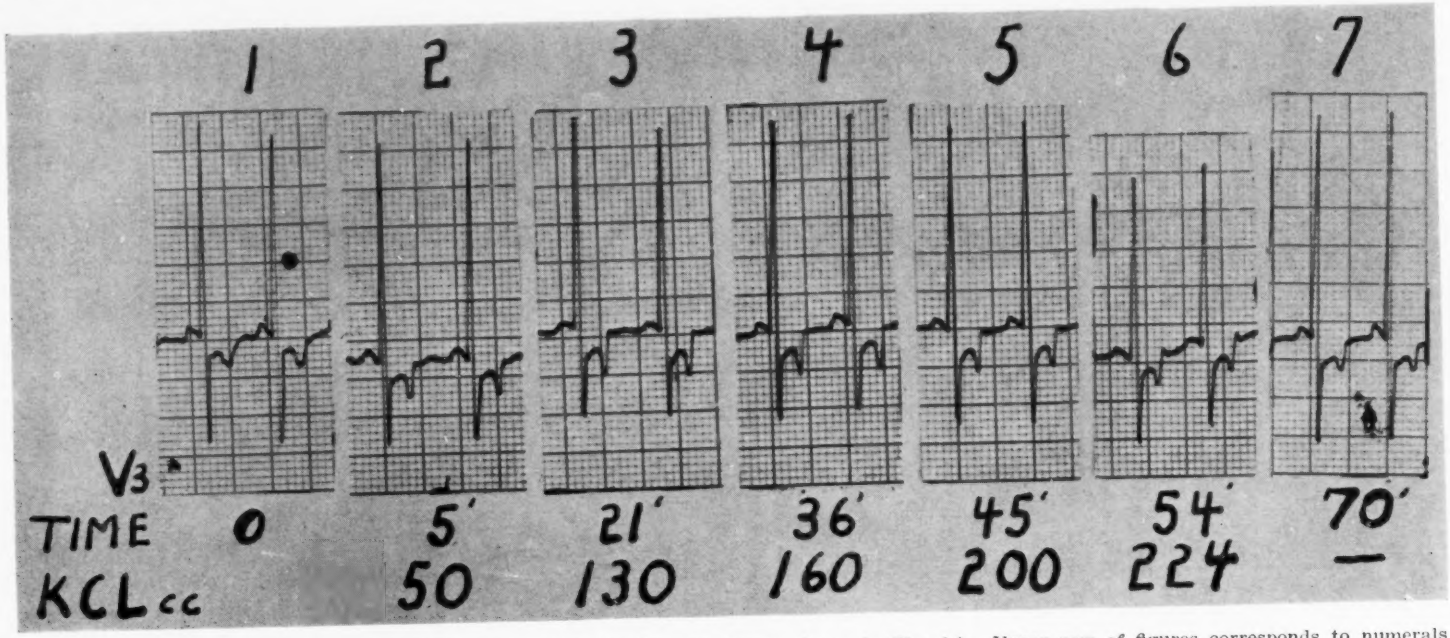

Fig. 3*B*.—Concomitant electrocardiographic tracings taken in the experiment shown in Fig. 3*A*. Upper row of figures corresponds to numerals on the kymographic tracing.

but the T wave became more inverted and the Q-T interval shorter. Sixteen minutes after the termination of the potassium infusion, the electrocardiogram had returned to the control.

DISCUSSION

In unobstructed dogs, the intravenous administration of potassium chloride did not result in demonstrable changes in intestinal motility. This was also the case for those obstructed animals which showed continued persistence of intestinal motility; it was only with those animals whose intestinal activity was markedly depressed that the stimulant effect of potassium chloride infusions became apparent. In these latter experiments all the animals improved clinically, becoming more alert with an improvement in muscular tone and external respiratory movements. In some instances defecation occurred. A point of corollary interest has been demonstrated by the work of Bellet and co-workers[1, 2] who have shown that, following intestinal obstruction, there occurred a deficit in serum potassium; this was corrected by the administration of potassium chloride. Also, Darrow[8] has shown that the administration of potassium chloride in infantile diarrhea resulted in a retention of potassium; this was taken to indicate the presence of a potassium depletion associated with the loss of large volumes of fluid.

The electrocardiographic changes occurring with the administration of potassium in the nonobstructed animal have been described previously by Winkler, Hoff, and Smith[9] and more recently by Bellet and associates.[10] An additional observation, noted in the present experiments, was that in two animals which had inverted T waves in the control, with the administration of potassium the T waves became more sharply inverted. This finding has been reported in dogs with myocardial infarction which had been given potassium.[10] However, an autopsy of the animals used in these experiments showed no evidence of myocardial infarction.

In two of the obstructed animals all of the typical electrocardiographic changes associated with clinical hypopotassemia occurred; these included S-T depression, Q-T lengthening, and T-wave inversion. The electrocardiographic changes in these two animals were associated with marked depression of the intestinal motility. In the remaining experiments, the most constant electrocardiographic finding was S-T depression.

An analysis of the electrocardiographic changes occurring with the administration of potassium chloride to the obstructed dog showed Q-T interval shortening to be the most constant finding. When potassium was administered to obstructed dogs with inverted T waves, there occurred sharp deepening of this wave. As previously stated, a similar response was obtained with unobstructed animals showing inverted T waves in the control.

In both animal and human experiments, the administration of potassium chloride was associated with stimulation of bowel motility. The administration of potassium may be an important adjunct in cases of paralytic ileus which may be due not only to intrinsic gastrointestinal disease but also to extrinsic

Fig. 3B.—Concomitant electrocardiographic tracings [illegible] on the kymographic tracing.

factors such as inflammation of the gall bladder or of the kidneys. However, potassium should be administered only if the serum potassium level is low or the general picture is that of hypopotassemia associated with paralytic ileus.

The electrocardiographic picture obtained in dogs given potassium chloride is not identical in all respects with that seen in human beings given potassium chloride. This may be due to the fact that the T waves often are very labile in dogs since the heart is subject to relatively extreme positional changes.

SUMMARY

Isotonic potassium chloride solution, by intravenous infusion, stimulates the intestinal motility in human beings and dogs with adynamic ileus associated with hypopotassemia.

Such an infusion has no effect on the intestinal motility of the normal dog as measured by the Thiry-Vella fistula preparation.

A few changes in the electrocardiogram of dogs do not correlate with human changes during the administration of potassium chloride.

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ZIRCONIUM TREATMENT IN IVY POISONING: PRELIMINARY INVESTIGATION

G. Arnold Cronk, M.D., and Dorothy E. Naumann, M.D.
Syracuse, N. Y.

ZIRCONIUM treatment of poison ivy dermatitis and its effectiveness in two experimental cases and forty-seven clinical cases are reported and discussed, with comment on toxicity of zirconium.

CLINICAL EXPERIMENT

Two female volunteers aged 15 and 55 years, both with history of several previous attacks of *Rhus toxicodendron* dermatitis, served in a preliminary experiment. A 1 to 10 dilution of *R. toxicodendron* extract was "applied" in drop form to three hairless areas on the volar aspect of the left forearm of each volunteer and allowed to dry. Immediately after drying, zirconium hydrate ointment was applied to the proximal area of the *R. toxicodendron* application; the middle and distal sites were left untreated.

The ointment was composed as follows:

Zirconium oxide	4.37%
Stearic acid	13.46%
Potassium, as KOH	1.86%
Glycerine	2.28%
Water and CO_2	78.03%

Glass containers were essential because the reducing effect of the zirconium on metal caused brownish discoloration of the ointment.

On the following day, small red papules appeared at the middle and distal sites. There was no evidence of dermatitis at the treated site of either volunteer. Zirconium hydrate ointment was then administered topically with moderate rubbing to the mid-site. On the second day after application, the proximal *R. toxicodendron* area continued to show no evidence of lesion, the mid-site was stationary and scaling, and a few vesicles had appeared at the distal site. One day later, the mid-site was brownish and scaling and definitely regressing. Several vesicles were apparent around site 3. On the next day no further progress of the dermatitis was noted at site 3.

CLINICAL APPLICATION

In our outpatient department and at a local camp during 1948 and 1949, forty-seven cases of dermatitis characteristically associated with R. *toxicodendron* contact were diagnosed, treated, and included in the study on the basis of (1) known recent poison ivy contact, (2) appearance of characteristic dermatitis, and (3) history of previous attacks of *R. toxicodendron* dermatitis. This type of dermatitis is so well known that controls are not deemed necessary or desirable to evaluate results.

From the Student Health Service, Syracuse University.
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All patients were treated similarly with the experimental preparation already described and were cautioned against further contact with *R. toxicodendron* and against use of any other therapeutic agent. All patients were asked to return daily for evaluation. Criteria of improvement were (a) relief of pruritis, (b) no extension of dermatitis, and (c) regression of existing dermatitis.

SAMPLING OF CASE HISTORIES

The following are typical of the forty-seven cases.

CASE 1.—A 27-year-old man with previous history of *R. toxicodendron* dermatitis. Typical macular and vesicular dermatitis was prevent on hands and arms. *R. toxicodendron* contact had occurred three days previously. Moderate burning and pruritis were present. He was treated with zirconium hydrate ointment twice daily.

Four days after treatment the lesions had faded to brownish discoloration of the skin. The ointment had relieved pruritis and burning immediately. No further therapy was necessary.

Treatment was effective.

CASE 2.—A 22-year-old man reported poison ivy contact two days previously while on field trip. On the evening prior to observation, the patient developed macular rash on the forearm and chest which became vesiculated in a few hours. The lesions were typical of *R. toxicodendron* dermatitis. He was treated with zirconium hydrate ointment twice a day.

On the second day following treatment, all lesions were drying without weeping or pruritis. The disease was under control and no further therapy was used.

Treatment was effective.

CASE 3.—A 21-year-old man. Contact while swimming, five days prior to observation. When seen, he exhibited typical itching macular and vesicular dermatitis of three days' duration on the feet. He reported previous *R. toxicodendron* dermatitis. He was treated with zirconium hydrate ointment applied by dressing.

On the following day weeping and itching had subsided. One day later lesions were dry and under control. No further therapy.

Treatment was effective.

CASE 4.—A woman with previous history of *R. toxicodendron* dermatitis. She reported poison ivy contact seven days previously. Three days after contact a macular rash on the feet and legs had appeared; five days after contact, lesions had increased in number with some vesiculation and moderate pruritis. She was treated with zirconium hydrate ointment twice a day. Two days after treatment the lesions were dry and desquamating; there was no pruritis. No further treatment was necessary.

Treatment was effective.

CASE 5.—A 26-year-old man with a history of previous *R. toxicodendron* dermatitis and known poison ivy contact three days before observation. On the day of observation, a macular vesicular rash developed on face and arms. He was treated with zirconium hydrate ointment twice a day.

Two days after treatment the lesions on the face were dry and desquamating, but the lesions on the arms were spreading with weeping and burning. Treatment with zirconium hydrate ointment was continued.

One day later the lesions on the arms and hands were much worse, with moderate weeping and spreading of vesicles. He was treated with Ivyol, 1 ampule, and wet Burrows packs 1:2,000. Twenty-four hours later there were no new lesions, no pruritis, and weeping had improved. The next day the lesions were dry and desquamating and the disease was under control. Patient treated with 1 ampule Ivyol.

Treatment with zirconium hydrate ointment was not effective.

TABLE I. EFFECTS OF TREATMENT OF RHUS TOXICODENDRON DERMATITIS WITH ZIRCONIUM HYDRATE OINTMENT

DAYS ELAPSED, CONTACT TO THERAPY:	1	2	3	4	5	6	7 OR MORE	TOTALS NUMBER	TOTALS PER CENT
Improved within 24 hours after therapy (cases)	10	9	13	1	1	1	4	39	85
Unimproved after zirconium therapy (cases)	1	2	3	0	1	0	0	7	15
								46	100

(Omitting one case, improved forty-eight hours after therapy.)

Table I summarizes the forty-seven clinically treated cases.

The foregoing data indicate highly satisfactory results in the treatment of the *R. toxicodendron* type of contact dermatitis with zirconium. Within twenty-four hours after starting therapy, 85 per cent of the patients showed relief of pruritis and regression of the dermatitis. Only 15 per cent followed the familiar pattern of *R. toxicodendron* dermatitis. The stability of these percentages will be appraised as greater numbers of cases are observed and reported.

DISCUSSION

Efforts to identify the toxic factor responsible for development of characteristic vesiculating dermatitis in certain persons following contact with *R. toxicodendron* have led to varied researches. Until 1934 the causative agent was believed to be an oleoresin; then Hill, Mattacotti, and Graham[1] demonstrated that urushiol is the toxic principle of *R. toxicodendron*. Urushiol is an ortho-dihydroxy-benzene containing an unsaturated hydrocarbon substitute in number 3 position. Experiments by these men[1] and by Toyama[2] prove that the hydroxyl groups in the urushiol are the chief cause of the well-known vesicant reaction. Further investigations[3-6] verify the conclusion that urushiol, a phenolic compound, is the active ingredient in *R. toxicodendron*.

The vesicant activity of urushiol depends on two properties: first an oleoresin-like characteristic that enables it to penetrate skin; second, the dihydroxy-benzene nucleus which provides the irritant action. It is thought that the long hydrocarbon side-chain increases its solubility in the oils of the skin. The characteristic symptoms of "ivy poisoning" appear once this substance penetrates the skin.

The multitude of remedies for treatment of this dermatitis indicate vividly the difficulty of its control. It has been assumed that once the toxin has fixed itself in the skin the reaction is irreversible. Treatment, therefore, either must be directed to preventing the fixation of this substance in the skin (most contemporary treatment is so oriented) or there must be some form of replacement therapy to remove or neutralize the toxin and thereby terminate its action.

Working in the laboratories of the Titanium Alloy Corporation, and aware of the properties—especially its replacement ability—of zirconium, Dr. E. Wainger conceived the idea that a suitable zirconium compound might combine with the hydroxyl groups of urushiol to render it inactive. He obtained an inactive precipitate on adding a solution of zirconium salts to urushiol, and further testing of the filtrate revealed absence of organic material. Applied to

susceptible volunteers, the filtrate did not effect the vesicant reaction. He concluded that the zirconium had broken down the urushiol and precipitated the toxic component.[7]

TOXICITY

The potentialities of zirconium, both as a possible industrial poison and as a useful pharmacologic agent, have been investigated on a limited scale. Fairhall[8] examined the possibilities of industrial hazards in connection with inorganic salts of zirconium. From a medical point of view, he discovered no evidence of local or systemic poisoning from these salts. His explanation was based on the insolubility of many zirconium compounds and their consequent poor absorption into the animal body. Together with the similarity of zirconium to bismuth in certain physical properties, this fact permits the use of zirconium oxide as a contrast medium in diagnostic x-ray examination of the gastrointestinal tract.

In further studies Fairhall observed the parenteral administration of zirconium salts. He found that animals tolerate large doses (in rabbits, 1.5 to 2 mg. per kilogram of body weight) without lethal effect. Harding[9] verified these findings and concluded that zirconium is physiologically inert.

In 1936 Gould[10] studied the effects of several heavy metals, including zirconium, in enzyme actions. With the possible exception of blood phosphatase, which was inactivated completely by zirconium salts, this heavy metal was essentially inert in vitro.

Another application of zirconium may be in connection with the extensive scientific investigation of radioactive metals, which presents a very pronounced new hazard to laboratory workers. At the Arizona National Laboratories, Schubert[11] studied this problem actively in laboratory animals. With a theoretic background of ion displacement, he found that animals experimentally poisoned with radioactive plutonium increased the urinary excretion of Pu^{239} following parenteral administration of zirconium salts. Zirconium in doses of 1.5 to 2.0 mg. per kilogram of body weight produced no harmful effect.

Our experience with topical application of zirconium hydrate in an ointment has been devoid of toxic manifestation either locally or from potential absorption.

CONCLUSIONS

In thirty-nine of forty-seven cases application of zirconium hydrate to *R. toxicodendron* dermatitis appeared to alter and shorten the duration of the dermatitis.

In a limited control study, early application of zirconium hydrate apparently was able to prevent the development of *R. toxicodendron* dermatitis.

None of the forty-nine cases in which zirconium hydrate was applied topically yielded evidence of topical or systemic toxicity.

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THE IDENTIFICATION OF INFLUENZA VIRUS ANTIBODIES BY MEANS OF THE COLLODION PARTICLE AGGLUTINATION REACTION

A. V. Boand, M.S., and J. E. Kempf, M.D.
Chicago, Ill.

THE collodion particle agglutination technique as a serologic method has been employed by numerous workers[1-5, 8] using a wide variety of antigens and antibodies. Most of these workers found that, although the method was extremely sensitive, nonspecific reactions were likely to occur. The method has, therefore, not been used commonly. The main reason for the failure of the technique was probably the inability to obtain uniformly active particles. Cavelti's[3] modification of the original method by Cannon and Marshall[1] seemed to produce effective particles as he reported satisfactory results in his studies with tissue antigens and antibodies. Lange and co-workers,[5] however, using particles prepared by a slight modification of Cavelti's method, did not obtain entirely satisfactory results in their study on autoantibodies of human glomerulonephritis. The reports of Saslaw and Campbell[4, 6] are encouraging, however. Using particles prepared by Cavelti's method these workers have had good results in their study with the histoplasmin antigen; they stress the simplicity and precision of the method.

Goodner[2] studied virus agglutinations using the collodion particle method and reported some success. He stated that the method could be successfully used for the identification of the yellow fever and poliomyelitis viruses, for the typing of the influenza virus, and for the detection of antibodies to these viruses in the serum of convalescent patients. Positive results were obtained with sera of human and caprine origin, though sera from rabbits and monkeys were negative. Pearson[7] failed, however, to duplicate Goodner's work using serum from man and ferrets with two strains of the influenza virus grown in the allantoic sac of the embryonated egg, and he also failed to observe agglutination with the Lansing strain of poliomyelitis virus and immune serum from monkeys. Nonspecific reactions were common with the virus systems studied.

In spite of the reported nonspecific reactions in the early work with the collodion particle agglutination method, investigators who have worked with the technique feel that it offers definite possibilities for the detection of antibodies, especially where relatively weak concentrations must be employed and where the conventional serologic methods lack the needed sensitivity. Because of these possibilities it seemed desirable to study the various factors affecting the reaction and to determine whether the test could be applied to the study of virus antivirus systems.

The present study was confined to the use of the influenza virus which was tested with human, rabbit, and guinea pig sera.

From the Department of Bacteriology, College of Medicine, University of Illinois.

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METHODS

Regardless of the antigen used in the collodion particle agglutination method, the procedure in the test is similar. The antigen must be adsorbed onto the collodion particles, thus sensitizing them. On adding specific antiserum followed by centrifugation the sensitized particles adhere and clump together. In spite of the apparent simplicity of the test there are many factors associated with the antigen, antiserum, collodion particles, and diluent which must be controlled properly to ensure consistent results. A thorough study of these factors was considered essential if nonspecific reactions were to be avoided.

(a) *Preparation of the Collodion Particles.*—

The importance of uniformly active particles cannot be exaggerated. The method as described by Cavelti has proved very successful, as nonspecific reactions due to the particles themselves have been eliminated by this method. Saslaw and Campbell[6] reported good results with these particles and stressed the stability of particles prepared by this method. Particles prepared by the simpler method of Havens and Lloyd[8] were also satisfactory although they were less stable.

It was advantageous to prepare particles in smaller batches than those described by Cavelti inasmuch as particles over four months of age were unsatisfactory. Stock suspensions were made to match the McFarland No. 2 nephelometer standard[19] when diluted 1:20. One part of stock suspension to nine parts of antigen suspension usually gave the desired concentration.

(b) *Preparation of the Antigen.*—

The virus antigens used in this work were prepared from Formalin-killed PR8 influenza virus which had been grown in the allantoic sac of the embryonated egg. Much of the chick protein was first removed by the red blood cell adsorption and elution technique,[10] and the virus was then concentrated by high-speed centrifugation in an angle-head centrifuge* at 18,000 r.p.m. (25,000 g) for one hour at 0 to 2° C. Concentrated virus was then suspended in 0.1M phosphate buffer at pH 7.0. A final centrifugation at 2,500 r.p.m. for five minutes was then carried out to remove visible particulate matter, which had been found to be a frequent cause of nonspecific reactions.

(c) *Sensitization of the Particles.*—

In preparing the sensitized particles a dilution of 1:10 was made of the stock suspension of collodion particles in a virus suspension. Adsorption took place within fifteen minutes at room temperature. Equal volumes of sensitized particles were then added to the serum dilutions resulting in a final concentration of collodion particles of one-twentieth that of the stock solution.

d. *The Diluent.*—

The diluent for testing human antiserum was 1.0 per cent saline prepared from chemically pure NaCl and triple distilled (in glass) water. The use of triple distilled water is recommended for all reagents since occasional nonspecific reactions occurred when singly distilled water was used.

(e) *The Serum.*—

Human sera was used for the most part in this study although rabbit and guinea pig sera were also used and found satisfactory. Serum was heated at 56° C. for thirty minutes prior to use, since occasional nonspecific agglutinations were traced to sera which had not been heated.

The human sera used were collected from persons having positive and negative histories of influenza, and a limited amount of sera was obtained from persons vaccinated with influenza antigen. Difficulty was encountered in finding persons with sera containing no influenzal antibodies, though such sera were obtained from young children with negative histories of influenza.

(f) *Procedure for the Test.*—

Serum dilutions were prepared in saline in doubling dilutions ranging from about 1:5 to beyond the suspected end point, which in a few instances was as high as 1:10,240. Three

*International Equipment Co., four-place multispeed attachment #S17167.

tenths milliliter of the serum dilutions was added to chemically clean tubes having an inside diameter of 7 mm. and length of 70 mm. Tubes of this size were the most satisfactory. Following the addition of the serum to the tubes, 0.3 ml. of the sensitized particles was added to the tubes. After mixing, a period of incubation was allowed to ensure maximum reaction although, as described later, the reaction occurred rapidly and the incubation merely effected a stronger agglutination instead of influencing the titration end point.

Since visible flocculation did not occur as the result of mixing the sensitized particles with immune serum, centrifugation of the tubes was necessary to bring about agglutination. Tubes were placed in an International No. 2 centrifuge and centrifugated at 1,500 r.p.m. for three minutes. The titration was then read by holding each tube against a black screen with a light source slightly above and behind and gently resuspending the sediment from the bottom of the tube by gentle shaking. Negative tubes were characterized by a homogeneous turbidity on resuspension, whereas positive tubes contained particles of varying sizes.

(g) *The Control System.*—

It was essential to control all components alone and in combination with others inasmuch as any component might have had a tendency for nonspecific reactions, and a combination of the various components would have increased this tendency. In this work eight controls were used. It was not essential to run all of them each time, but each combination was tested to determine the likelihood of nonspecific reactions. The following controls were used:

1. Unknown serum (lowest dilution) + particles.
2. Normal serum (lowest dilution) + particles.
3. Normal serum + sensitized particles.
4. Control antigen + particles and saline.
5. Control antigen* and particles + immune serum (lowest dilution).
6. Control antigen and particles + normal serum (lowest dilution).
7. Sensitized particles + anticontrol antigen serum.
8. Collodion particles + saline.

EXPERIMENTAL AND RESULTS

(a) *The Effect of the Antigen.*—

Probably more nonspecific reactions are due to the antigen than to any other component, since particle size, impurities in excess, pH variations, or inadequate concentrations will invariably lead to poor results.

In order to obtain maximum titrations of serum, the optimal antigen concentration necessary to properly sensitize the particles was determined. Various antigen concentrations were titrated with antisera and the concentration selected was that which gave the highest titer, strongest agglutination, and the fewest nonspecific reactions. As shown in Table I, an influenza antigen with a CCA titer[12] of 1:5,120 produced maximum sensitization of the particles. Higher or lower concentrations showed lower titration values. Removal of antigen excess after adsorption may be done by centrifuging the sensitized particles and then resuspending them in buffer solution. However, this procedure tended to increase the incidence of nonspecific reactions.

Many routine assays of antisera were carried out using a commercial influenza vaccine as antigen.† This vaccine had been prepared in a manner similar to that already described and proved satisfactory if centrifuged to remove visible particulate matter. The concentration of this antigen was not suffi-

*Normal allantoic fluid prepared by same method as the viral antigen.

†Obtained through the courtesy of E. R. Squibb & Sons.

TABLE I. DETERMINATION OF THE OPTIMAL CONCENTRATION OF INFLUENZA VIRUS ANTIGEN FOR COLLODION PARTICLE AGGLUTINATION USING HUMAN ANTI-INFLUENZA SERUM

CCA TITER OF ANTIGEN BEFORE MIXTURE WITH EQUAL QUANTITY OF COLLODION PARTICLES	SERUM DILUTIONS										CONTROLS*	
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	ANTIGEN	SERUM
160	0	0	0	0	0	0	0	0	0	0	0	0
320	+	+	0	0	0	0	0	0	0	0	0	0
640	+	+	+	0	0	0	0	0	0	0	0	0
1,280	+	+	+	+	+	+	0	0	0	0	0	0
2,560	+	+	+	+	+	+	+	0	0	0	0	0
5,120	+	+	+	+	+	+	+	+	+	0	0	0
10,240	+	+	+	+	+	+	+	+	0	0	0	0
25,000	+	+	+	+	+	+	+	0	0	0	0	0

*In antigen control, sensitized particles were mixed with 1:10 dilution of normal serum.
In serum control, unsensitized particles were mixed with 1:10 dilution of immune serum.

TABLE II. THE EFFECT OF pH OF THE ANTIGEN ON COLLODION PARTICLE AGGLUTINATION

pH OF BUFFERED ANTIGENS*	POSITIVE SERUM DILUTIONS 0.3 ML.								CONTROLS†	
0.3 ML.	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	ANTIGEN	SERUM
5.0	+	+	+	+	+	+	+	+	+	+
6.0	+	+	+	+	+	+	+	+	+	+
6.5	+	+	+	+	+	0	0	0	0	0
7.0	+	+	+	+	+	+	+	0	0	0
7.5	+	+	+	+	0	0	0	0	0	0
8.0	+	0	0	0	0	0	0	0	0	0
9.0	0	0	0	0	0	0	0	0	0	0

*Influenza PR8 virus with CCA titer of 1:1,280 suspended in 0.1M phosphate buffer at indicated pH.
†Controls as in Table I.

TABLE III. EFFECT OF VARYING CONCENTRATIONS OF NaCl DILUENT ON THE COLLODION AGGLUTINATION METHOD USING INFLUENZA VIRUS ANTIGEN (CCA TITER 5,120)

SALINE CONCENTRATION	POSITIVE SERUM DILUTION										CONTROLS*	
(%)	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	ANTIGEN	SERUM
0.1	0	0	0	0	0	0	0	0	0	0	0	0
0.25	+	+	+	+	+	0	0	0	0	0	0	0
0.5	+	+	+	+	+	+	0	0	0	0	0	0
1.0	+	+	+	+	+	+	+	+	+	+	0	0
2.5	+	+	+	+	+	+	+	+	0	0	0	0
5.0	+	+	+	+	+	+	+	0	0	0	0	0
7.5	+	+	+	+	+	+	0	0	0	0	+	0
10.0	+	+	+	+	+	0	0	0	0	0	+	0
20.0	0	0	0	0	0	0	0	0	0	0	0	0

*Controls as in Table I.

ciently high (1,280 CCA units) for maximum sensitization of the particles, thus titers were somewhat lower. This antigen was desirable for comparative assays, however, because of its convenience and economy.

(b) *The Effect of pH and Molar Concentration of Electrolyte in the Diluent.*—

As pointed out by Cavelti the satisfactory pH range of the antigen is relatively narrow, and nonspecific reactions readily occur outside that range. Table II demonstrates the effect of pH; specific agglutinations occurred only when the virus antigen was buffered between pH 6.5 and 7.5 and greatest sensitivity occurred at pH 7.0.

Molarity of the buffer was of lesser importance since satisfactory results were obtained with phosphate buffers ranging up to 0.5M, provided the pH was at 7.0.

(1) *The Effect of Diluent Concentration:* The effects of varied concentrations of saline diluent are shown in Tabel III. Using human serum 1.0 per cent saline proved most satisfactory although agglutinations occurred with concentrations varying from 0.25 per cent to 10 per cent. In concentrations above 5.0 per cent, however, titers were greatly diminished and were not considered reliable. A 20 per cent saline solution inhibited agglutination completely.

(2) *The Effect of Valency of Electrolyte:* Because of the inconvenience of centrifuging the particles to bring about agglutination, the use of electrolytes other than NaCl as diluents was tried in the hope of altering the potential difference (PD) between the particles and the diluent, and thus bringing about flocculation. Loeb[11] in his paper on the theoretical basis for the collodion particle agglutination showed that the particles were negatively charged and that the normal cataphoretic PD between the particles and the dispersion fluid was about 70 millivolts. Since agglutination of the particles occurs when the PD falls below a critical point (about 16 millivolts), electrolytes with di- and trivalent cations were employed to depress the PD. This, however, proved entirely unsatisfactory as all di- and trivalent electrolytes tested brought about flocculation of the protein in both antigen and serum.

(c) *The Effect of Time and Temperature of Incubation.*— Since most workers have recommended an incubation period of from one to two hours for adsorption of the antigen onto the particles, studies were carried out to determine the necessity for this procedure. It was found that adsorption took place rapidly and that no better results could be obtained by incubation for one or two hours than by incubation for fifteen minutes at room temperature. At 4° C., however, adsorption was slow and overnight incubation was required. Lower titers were consistently obtained by incubation at 37° C. than were obtained at room temperature or at 4° C., indicating less adsorption at 37° C.

Some forty experiments on the time of incubation necessary for agglutination after mixing the sensitized particles and immune serum revealed that agglutination could be brought about immediately after mixing. Maximum agglutination was obtained following thirty minutes at room temperature. Longer periods increased the intensity of the reaction but did not affect the sensitivity. Incubation periods of one to two hours occasionally caused the

TABLE IV. TITRATION OF HETEROLOGOUS VIRAL SERUM WITH INFLUENZA PR8 ANTIGEN (CCA TITER 2,560)

IMMUNE HUMAN SERA	BEFORE ABSORPTION WITH INFLUENZA A AND B VIRUS — SERUM DILUTIONS									AFTER ABSORPTION WITH INFLUENZA A AND B VIRUS	
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:10	1:20
Western equine encephalitis	+	+	+	+	0	0	0	0	0	0	0
Western equine encephalitis	+	+	+	0	0	0	0	0	0	0	0
Psittacosis-lymphogranuloma venereum	+	+	+	0	0	0	0	0	0	0	0
Psittacosis-lymphogranuloma venereum	+	+	+	+	+	+	+	0	0	+	0
Psittacosis-lymphogranuloma venereum	+	+	0	0	0	0	0	0	0	0	0
Lymphocytic choriomeningitis	+	+	+	+	0	0	0	0	0	0	0
Lymphocytic choriomeningitis	+	+	0	0	0	0	0	0	0	0	0
Japanese B encephalitis	+	+	+	+	0	0	0	0	0	0	0
Japanese B encephalitis	+	+	0	0	0	0	0	0	0	0	0
Influenza A and B (control)	+	+	+	+	+	+	+	+	0	0	0
HYPERIMMMUNE GUINEA PIG SERA											
Western equine encephalitis	0	0	0	0	0	0	0	0	0		
Lymphocytic choriomeningitis	0	0	0	0	0	0	0	0	0		
Normal guinea pig	0	0	0	0	0	0	0	0	0		

appearance of nonspecific reactions which were avoided by shorter periods of incubation. Incubation at 4° and 37° C. were less satisfactory than at room temperature since maximum titers were not obtained.

A device for shaking the tubes during incubation was employed in the hope that bringing the particles into greater contact would eliminate the need of centrifugation in bringing about agglutination. Flocculation was observed in sera of low dilution, but a precise end point could not be obtained by this process.

(d) *The Effect of Centrifugation of the Particles.*—

Centrifugation was relied on to bring about agglutination inasmuch as other methods were unsuccessful. A study of the various speeds of centrifugation showed that 1,400 to 1,500 r.p.m. for three minutes was adequate. Higher speeds resulted in difficulty in resuspension.

(e) *The Sensitivity and Specificity of the Method.*—

In order to learn more of the specificity of the technique, a limited number of heterologous human immune sera* was tested with the influenzal antigen. As controls, hyperimmune and normal guinea pig sera were included. No high titer cross reactions against the influenzal antigen were observed in these sera (Table IV), although in all human sera influenzal antibodies were demonstrated in the titers normally found in adult sera. Specificity was demonstrated by the removal of the influenzal antibodies by adsorption with influenzal virus. Hyperimmune guinea pig sera against WEE and lymphocytic choriomeningitis showed no agglutination.

It was observed that in antiserum produced by vaccination, antibodies against both the virus and the chick protein were detected. The difference in titer was significant, however, since the chick protein titer was approximately 1:40, whereas the viral titer was usually 1:1,280, or about thirty times that of the chick protein.

Comparison of the sensitivities of the collodion particle and the hemagglutination inhibition method[12] were also carried out. The data in Table V indicate that the collodion particle method was eight times more sensitive than the H-I method.

(f) *The Effect of Preservation of Serum.*—

Cavelti reported that human sera showed an increased tendency for nonspecific reaction on storage. Our findings, however, failed to show that this held true with the virus system studied. Sera were stored for as long as one year at –30° C. with unaltered specificity provided they were not contaminated. The addition of 0.3 per cent phenol or of 1:10,000 Merthiolate as a preservative did not seem to alter the reaction.

(g) *The Interpretation of Controls.*—

In running the aforementioned controls it was found that nonspecific agglutinations rarely occurred. Fatty serum, however, was found to be a fre-

*We are indebted for these sera to Dr. Joel Warren, Department of Virus and Rickettsial Diseases, Army Medical Department, Research and Graduate School, Army Medical Center, Washington, D. C.

TABLE V. COMPARISON OF SENSITIVITIES OF THE COLLODION PARTICLE METHOD WITH THE HEMAGGLUTINATION INHIBITION METHOD USING INFLUENZA PR8 VIRUS (CCA TITER 5,120)

	POSITIVE SERUM DILUTIONS		
HUMAN SERUM	HEMAGGLUTINATION INHIBITION TITRATION END POINT	COLLODION PARTICLE AGGLUTINATION TITRATION END POINT	SENSITIVITY DIFFERENCE
1	1:320	1:2,560	8×
2	1:320	1:2,560	8×
3	1:320	1:1,280	4×
4	1:160	1:1,280	8×
5	1:160	1:1,280	8×
6	1:160	1:1,280	8×
7	1:160	1:1,280	8×
8	1:160	1:1,280	8×
9	1:80	1:640	8×
10	1:80	1:640	8×
11	1:80	1:640	8×

quent cause of such reactions, and grossly hemolyzed serum occasionally was the cause. The collodion particle and saline control tube frequently showed a granular flocculation which was easily differentiated from the viral type of agglutination. The presence of a stabilizing substance such as normal or immune serum, even in very high dilutions, prevented this flocculation.

DISCUSSION

Numerous workers have emphasized the specificity and high degree of sensitivity of the collodion particle agglutination technique but, since others have observed the frequent occurrence of agglutinations due to other factors than the antigen-antibody reaction, the method has not been used widely.

In these experiments using the influenzal virus and homologous antiserum it was found that the technique was sensitive and specific providing the various factors affecting the reaction were controlled. The method was simple and rapid, and once the procedure was standardized for a particular purpose it seemed at least as free of difficulties as any of the present serologic methods. Extensive study on a comparison of these methods seems indicated.

Critical factors were pH and the concentration and purity of the antigen. Although the use of a rather concentrated antigen was necessary to obtain the highest degree of sensitivity, less concentrated antigen was used to advantage in routine serum titrations. The method was standardized with this antigen and yielded consistent although somewhat lower titrations. A commercial influenza vaccine was used extensively for these comparative titrations.

While purity and concentration of the antigen were of primary importance, other factors such as the diluent used, the time and temperature of incubation, and the treatment of the serum were of lesser importance. Specific agglutinations were observed over a wide range of saline concentrations although 1 per cent saline proved most satisfactory. Incubation at room temperature gave the most satisfactory results; incubation at either 4° or 37° C. lessened the sensitivity of the reaction. Prolonged incubation periods were of no value and nonspecific reactions were frequently attributed to long incubation periods.

In spite of the fact that many workers have discarded the collodion particle agglutination method because of nonspecific reactions, the method as used in these studies appears to provide a useful tool for the studies of viral antibodies. Because of its sensitivity, specificity, and simplicity the method seems potentially valuable in viral diagnosis and in epidemiologic studies.

SUMMARY

A method for titrating the influenzal virus antibodies by the use of the collodion particle agglutination technique of Cavelti is described in detail. The various factors influencing the reaction were taken into consideration as were the means by which nonspecific reactions could be eliminated. A concentration of collodion particles of one-twentieth that of a McFarland No. 2 nephelometer standard, a CCA titer of virus from 1:1,280 to 1:5,120, a pH of the diluent of 7.0, and NaCl concentration of 1 per cent were necessary for specific, sensitive, and reproducible results. Formalinized virus was a stable and dependable antigen for use in the test.

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THE PROTECTION OF INTRACELLULAR BRUCELLA AGAINST STREPTOMYCIN ALONE AND IN COMBINATION WITH OTHER ANTIBIOTICS

ROBERT L. MAGOFFIN, M.D., AND WESLEY W. SPINK, M.D.
MINNEAPOLIS, MINN.

IT IS now well established that during the course of experimental brucellosis in animals, brucella occur intracellularly in the parenchymal tissues and in circulating phagocytes. Smith[1] early called attention to intracellular parasitism by brucella when he demonstrated the characteristic localization of enormous numbers of these organisms in the cytoplasm of the chorionic epithelium in infected fetal membranes from aborting cows and goats. Subsequent studies by Goodpasture and Anderson[2] and Buddingh and Womack[3] utilizing the chick embryo showed the invasion and multiplication of brucella within cells of the vascular endothelium, spleen, liver, kidneys, and myocardium. Other observers have demonstrated intracellular brucella in experimentally infected guinea pigs, rabbits, and mice.[4, 5, 6]

The observation of intracellular brucella in man has been reported by Meyer[7] who demonstrated clusters of the organisms in epithelial cells of the proximal tubules and glomerular capsule in sections of kidney in a fatal case of brucellosis. In addition to these observations it may be assumed that in cases of bacteremia due to brucella, as in other bacteremias, many of the organisms in the peripheral circulation occur intracellularly within leucocytes as a result of phagocytosis.

If intracellular parasitism is a constant feature in the pathogenesis of brucellosis, it is of practical importance to determine the extent to which organisms situated intracellularly are vulnerable to the action of available chemotherapeutic agents. In 1916, Rous and Jones[8] working with typhoid bacilli found that phagocytized organisms were protected from the lethal action of chemical poisons such as potassium cyanide and also from the bactericidal action of specific antiserum. They further showed that protection from these agents was dependent upon living phagocytes. Upon death of the phagocytic cell, even though the cell membrane appeared to remain intact, the intracellular organisms were no longer protected.

In recent years a number of chemotherapeutic agents which possess marked antibacterial action in vitro against brucella and other gram-negative organisms have proved to be of limited value in the therapy of infections caused by these bacteria. In the case of streptomycin this discrepancy has been explained by the apparent failure of the antibiotic to penetrate liver, spleen, and other tissues in therapeutic quantities even though adequate blood levels are achieved.[9]

From the Department of Medicine, University of Minnesota Hospitals and Medical School.
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Nelson, Forgacs, and Kucera[10] further suggested that therapeutic failures might well be due to the inability of antibiotics to reach organisms contained within the cells.

The present studies were carried out to determine if intracellular brucella are protected from the rapid lethal action of streptomycin, both when used alone and in conjunction with other antibiotics, particularly when the parasitized cells are exposed in vitro to high concentrations of antibiotics.

METHOD

A mixture containing intracellular brucella was prepared by adding 0.25 ml. of human blood with abundant leucocytes to 0.25 ml. of a heavy saline suspension ($BaSO_4$ series No. 1) of *Brucella suis*. To obtain a high concentration of leucocytes, 20 ml. of fresh venous blood drawn from an "immune" person (brucella agglutinin titer 1:640) were mixed with an equal volume of 1.0 per cent citrate in 0.4 per cent saline and centrifuged at moderate speed for ten minutes. The top milliliter of packed cells, including the buffy coat, was then carefully removed with a pipette. After incubation at 37° C. for forty-five minutes with occasional agitation, the mixture of buffy coat and brucella was diluted 1:10 in Trypticase-soy broth. Repeated examination of stained smears from this inoculum showed moderate phagocytosis with most of the leucocytes containing 3 to 10 organisms. It should be emphasized, however, that this inoculum still contained many extracellular organisms. An inoculum for control studies was prepared at the same time using similar amounts of buffy coat and brucella suspension, but by diluting the mixture immediately tenfold in broth without prior incubation no opportunity for phagocytosis was afforded.

Test tubes containing the desired concentration of antibiotic in 9.0 ml. of citrated (1.0 per cent) Trypticase-soy broth were inoculated with 1.0 ml. of the inoculum containing phagocytized brucella and incubated at 37° C. in a water bath. Initially and at suitable intervals for three to five days, 1.0 ml. portions were removed from each tube, diluted in tenfold series, and agar plate counts of the surviving organisms were made. Control tubes were similarly prepared and observed. After three to four days, rapid deterioration of the leucocytes was evident.

In one series of experiments an inoculum was prepared by grinding the spleens from mice infected with *Br. suis* five days prior to the experiment. Normal mouse spleen with an approximately equal number of brucella added was employed as a control inoculum in these experiments.

RESULTS

The effectiveness of streptomycin alone against phagocytized and extracellular brucella is compared in Fig. 1. It can be seen that there was rapid killing of the organisms in the control preparations so that within twenty-four hours or less no surviving organisms could be recovered. In contrast, the survival curves of the preparations containing phagocytized brucella show an initial drop representing the rapid killing of extracellular organisms after which there is only slight additional decline up to seventy-two hours. Similar results were obtained (Fig. 2) when ground mouse spleen from previously infected mice was used as the inoculum containing intracellular organisms.

When sodium sulfadiazine was added to streptomycin (Fig. 3) death of the intracellular organisms was still delayed, though the period of protection was shorter than when streptomycin alone was used. Similarly, protection of phagocytized brucella against combinations of streptomycin with aureomycin, chloramphenicol, and terramycin was observed (Figs. 4, 5, and 6). No constant

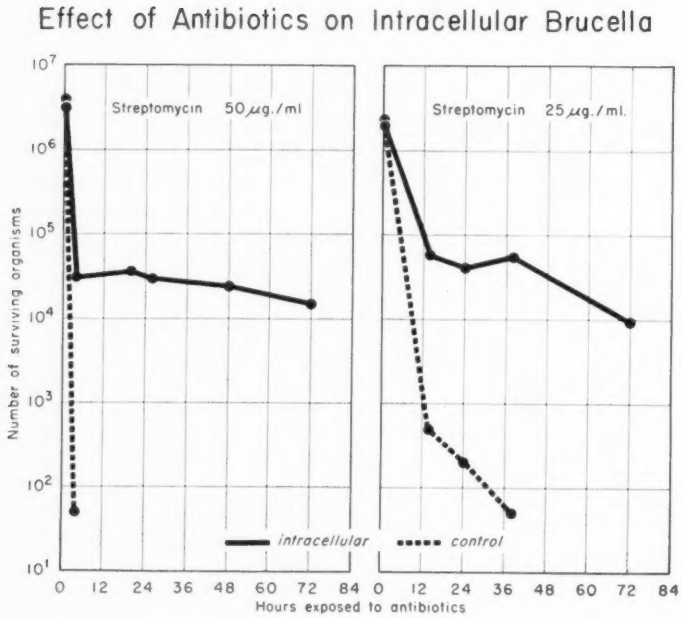
Effect of Antibiotics on Intracellular Brucella
Streptomycin 50 μg./ml.
Streptomycin 25 μg./ml.
Number of surviving organisms
intracellular
control
Hours exposed to antibiotics

Fig. 1.

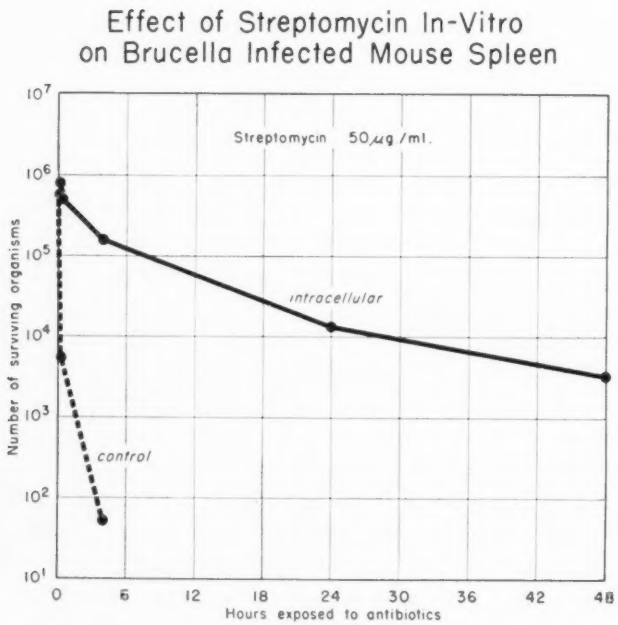
Effect of Streptomycin In-Vitro
on Brucella Infected Mouse Spleen
Streptomycin 50 μg./ml.
intracellular
control
Number of surviving organisms
Hours exposed to antibiotics

Fig. 2.

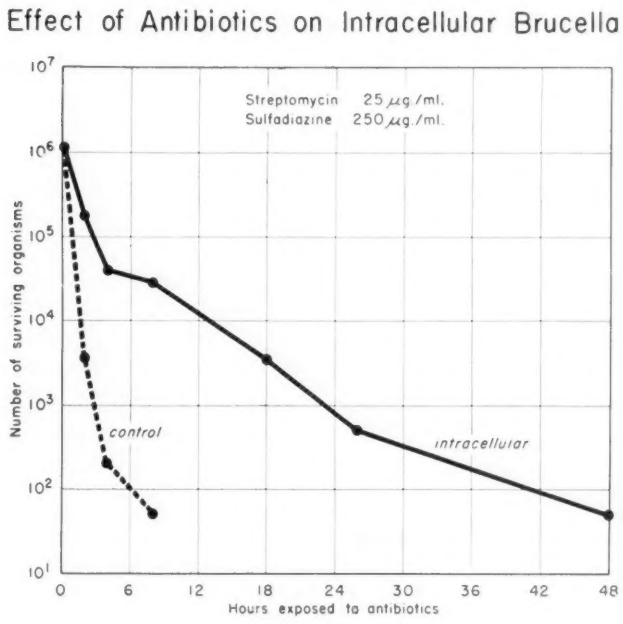
Effect of Antibiotics on Intracellular Brucella
Streptomycin 25 μg./ml.
Sulfadiazine 250 μg./ml.
control
intracellular
Number of surviving organisms
Hours exposed to antibiotics

Fig. 3.

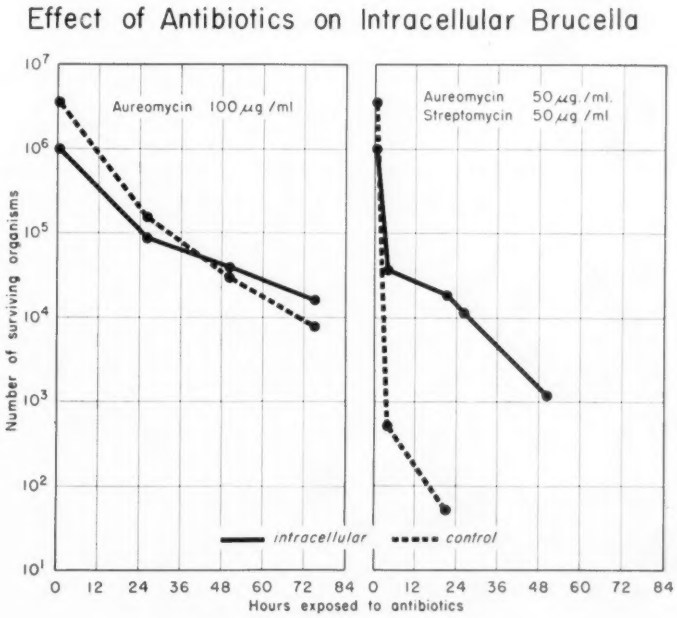
Effect of Antibiotics on Intracellular Brucella
Aureomycin 100 μg./ml
Aureomycin 50 μg./ml.
Streptomycin 50 μg./ml
intracellular
control
Number of surviving organisms
Hours exposed to antibiotics

Fig. 4.

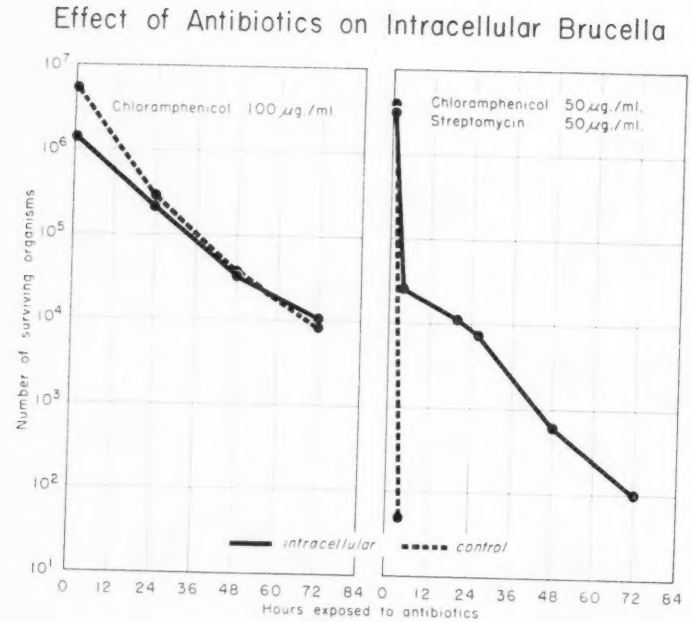
Effect of Antibiotics on Intracellular Brucella
Chloramphenicol 100 μg./ml.
Chloramphenicol 50 μg./ml.
Streptomycin 50 μg./ml.
Number of surviving organisms
intracellular
control
Hours exposed to antibiotics

Fig. 5.

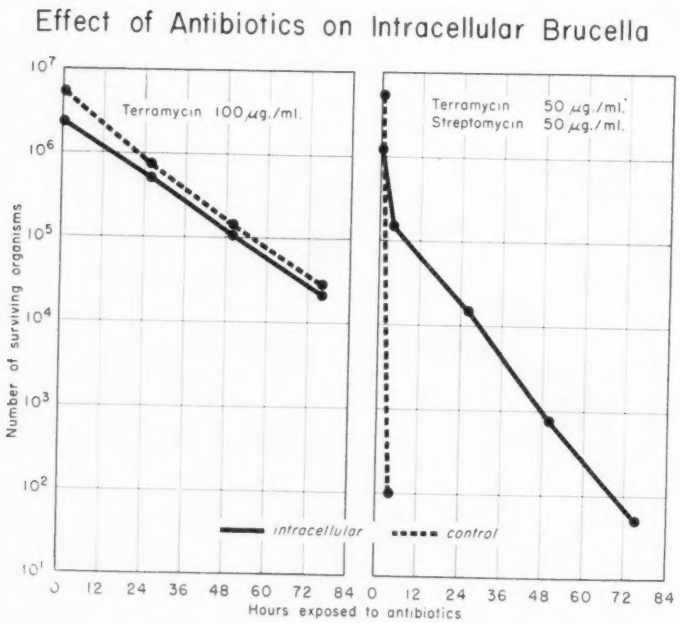
Effect of Antibiotics on Intracellular Brucella
Terramycin 100 μg./ml.
Terramycin 50 μg./ml.
Streptomycin 50 μg./ml.
Number of surviving organisms
intracellular
control
Hours exposed to antibiotics

Fig. 6.

differences in the survival curves of intracellular and extracellular brucella were observed when aureomycin, chloramphenicol, and terramycin were employed individually. Since the action of these antibiotics is primarily bacteriostatic even in the high concentrations employed, death of the extracellular organisms occurred too slowly to permit the demonstration of any possible protection of the intracellular organisms prior to death and disintegration of the harboring leucocytes.

COMMENTS

Though the conditions under which intracellular brucella were exposed to antibiotics in these experiments are artificial and cannot properly be compared to actual infection in vivo, the results do provide further information on the failure of streptomycin alone, or in combination with other agents, to eliminate completely organisms from the tissues, particularly when many of the bacteria are harbored intracellularly.

In view of numerous clinical studies reporting the value of streptomycin and sulfadiazine,[11-14] streptomycin and aureomycin,[15, 16] and aureomycin alone[17-20] in the therapy of human brucellosis, there is little cause to doubt that these agents effectively suppress the infection, relieve symptoms, prevent complications, and shorten the course of illness. However, the present study and the results of therapy with these agents in experimentally infected animals[21-26] emphasize the difficulty of completely eradicating brucella from the tissues. The evidence at hand suggests that the primary role of chemotherapy in brucellosis is general suppression of the infection and prevention of its spread to new foci, whereas final eradication of the organism is dependent to a large extent on the successful mobilization of the defenses of the host.

SUMMARY

The effect of streptomycin alone and in combination with sulfadiazine, aureomycin, terramycin, and chloramphenicol against intracellular and extracellular brucella was observed in vitro.

Protection of the intracellular organisms from the rapid lethal action of streptomycin alone and in conjunction with other antibiotics was observed.

No conclusions could be drawn from the results of these experiments as to the possible effect on intracellular brucella of aureomycin, terramycin, and chloramphenicol when used individually.

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AN EPIDEMIC OF TYPHOID FEVER AT SPENARD, ALASKA

WILLIAM H. GAUB, M.P.H., PH.D.*
ANCHORAGE, ALASKA

UNDIAGNOSED outbreaks of "gastrointestinal disturbance" occur frequently in Alaska. They comprise a baffling element of the disease control problem. Reports thereof are usually so long delayed that reliable information pertaining to the outbreaks is not always available. Laboratory specimens are seldom submitted if the affected individuals are located in isolated areas at some distance from medical and laboratory facilities. During a recent typical outbreak in Spenard, Alaska, all patients involved were fortunately located within an area easily accessible to medical, nursing, and laboratory services. The epidemic was therefore brought to medical attention at an earlier stage, which facilitated the collection of specimens and the development of a coordinated epidemiologic study.

The Spenard epidemic manifested several unusual characteristics considered worthy of reporting. Among them were the following: (1) the causal organism proved to be a strain of *Salmonella typhosa* untypeable by bacteriophagy; (2) a carrier with a similar strain was incriminated; (3) the distribution of cases involved children from 4 to 11 years of age, with no "teenage" children or adults; and (4) *Alcaligenes faecalis* was isolated from suspected cases, unconfirmed for *S. typhosa,* with typhoid-like symptoms. This organism was also isolated from stool specimens in a number of typhoid cases during the convalescent stage.

Spenard, the focal point of the recent epidemic, is an unincorporated and unrestricted utility district adjacent to Anchorage, the largest city in Alaska. In 1940, the homesteads in the Spenard area were subdivided for development and construction of low-cost and primitive housing. It became a utility district in 1946. The area comprises approximately 35 square miles with elevation ranging from approximately 150 feet to sea level. In it is situated the receding Lake Connell with its extensive marshy area. The population is mixed and comprises approximately 600 families with a population of 2,159.† As frequently occurs in a "boom" area, the general sanitary conditions are poor. Water is supplied from individual wells of which approximately 5 per cent are properly encased and located. Twenty-two per cent of all water samples collected during the past year in this district have shown some degree of contamination with confirmed coliform organisms. Approximately 5 per cent of homes have septic tanks; the balance have cesspools. Canned milk from outside sources is extensively used, although pasteurized milk from the Matanuska Valley producers is supplied to those families who can afford it.

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*Chief, Bacteriology and Parasitology Branch, Arctic Health Research Center, Public Health Service, Federal Security Agency.

†1950 population.

Many fresh and canned vegetables and fruits from Stateside sources are available in the local stores, with some vegetables and small fruits raised locally by individual families.

The present epidemic occurred in Spenard during the months of March and April, 1950. It involved seventeen persons, all between the ages of 4 and 11 years. The age distribution of these cases varied somewhat from the usual and currently reported age distribution pattern for the disease which generally involves teen-age children and young adults.[5] No adult cases or deaths occurred.

The strain of *S. typhosa* ultimately identified as responsible for this outbreak was bacteriophage typed as "Epidemic Strain—Untypeable Vi." An adult carrier with similar type of organisms, isolated from both stool and bile cultures, was incriminated after careful study. This individual had had typhoid fever in 1938, in the States, and had been responsible for at least one case in 1942, when she was employed as a cook in a private home. She had been registered as a carrier in 1942, with the Illinois State Department of Health, but subsequently moved to another jurisdiction and "became lost in circulation." She was employed for a short period of time in an eating establishment at Hanford, Wash., in 1943; she moved to Seattle, Wash., in 1944 and was employed as a janitoress. Residence was established in Spenard in the spring of 1949. She was employed for several weeks in an Anchorage restaurant in March, 1949; however, no cases of typhoid fever were ever traced to her in this employment. Her subsequent employment in Alaska was not as a public or commercial food handler.

It is difficult to indicate the actual date of onset of this epidemic since the mistaking of the protean nature of typhoidal symptoms among persons ill with undiagnosed diarrheal conditions possibly resulted in the "missing" of earlier cases. A number of the early cases were diagnosed as "intestinal flu," "virus infection," "flu," "fever of undetermined origin," and so forth, until laboratory work was undertaken in April to ascertain the possible causative etiology. From a review of case records of the six confirmed "primary" cases, the onset of symptoms occurred between March 10 and March 14, with "secondary" cases occurring between March 19 and April 20. The carrier and her son lived in a trailer adjacent to a family with five children, all except one of whom were "primary" cases. The fifth had had mild undiagnosed symptoms that cleared up before the epidemic occurred. The carrier's son was considered a "secondary" case, with the onset of symptoms dated March 20. There had been a free exchange of food between the families, social intercourse, and the use of the same well water supply and toilet facilities. Samples of this well water revealed confirmed coliform organisms upon at least one occasion in the early stages of the investigation. The custom among the children at school, and en route to school, of freely exchanging lunches, their frequent attendance at gatherings of children in organized groups where food was supplied by parents and freely exchanged among the children, the questionable health habits of some of the children, plus a generally poor sanitary environment probably contributed to the dissemination of the infection.

Immediately after the first case of typhoid fever had been confirmed, the usual preventive measures to control the epidemic were invoked by the Alaska Department of Health in cooperation with the Anchorage City Health Department.

The following specimens were obtained from each suspected case and contact for laboratory examination: stool specimens from each individual of at least three consecutive bowel evacuations were submitted to the laboratory in a selective preservative solution*; blood for culture,† if the patient had been febrile for three or more days with undetermined etiology; and blood for agglutinations.

A similar series of specimens was collected from each of forty-two persons residing in and about Anchorage who had had typhoid fever previously and had volunteered for check-up. From this latter group, a carrier with bacteriophage type "E_1" was detected. This individual was not aware of his carrier state although he had had typhoid fever in 1933. Due to the difference in type, this carrier was not incriminated.

Table I indicates the distribution of cases by sex and degree of severity of symptoms. Both sexes were involved. Male patients predominated over the female patients in an approximate ratio of 2.5:1.

TABLE I. DEGREE OF SEVERITY OF SYMPTOMS BY SEX

TOTAL CASES	TOTAL BY SEX		SEVERE		MODERATE		MILD OR SUBCLINICAL	
	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE
17	12	5	3	1	5	2	4	2

Sixteen persons with no immunization (94.1 per cent) within the previous two years succumbed to the infection. Mild or subclinical symptoms developed in a male subject within two years after completing a series of inoculations (5.9 per cent). Table II indicates the laboratory procedures involved in the confirmation of cases.

The Vi agglutination test, a screening test for the carrier state,[1] was performed upon the sera of all confirmed cases at least once during the illness

*_Stool preservative solution_: Sodium desoxycholate 0.5 per cent; sodium citrate (A. R. grade) 0.5 per cent; ferric ammonium citrate (green scales) 0.1 per cent in distilled water; pH 7.4. Dispensed 15 ml. in 10 dram screw-capped glass bottles. Exposed to flowing steam 20 minutes. Distributed with wood applicator stick and directions to inoculate bottle with fecal material "the size of a pea" and to include in bottle mucus and/or blood clots present in original specimen.

Upon receipt in the laboratory, the specimen was streaked upon Endo and SS agar plates; incubated 22 to 24 hours at 37° C. Typical group growth colonies were identified biochemically and serologically. Original specimen was incubated 22 to 24 hours at room temperature, streaked upon a second set of plates, and foregoing plate procedure repeated. After an additional 22 to 24 hours of incubation at room temperature, the specimen was streaked upon a third set of plates; the plate procedure was repeated. Original specimen was autoclaved and discarded.

†_Blood and bile culture media_: Bacto oxgall 10 per cent; proteose peptone 1 per cent in distilled water. Dispensed 15 ml. in 1 oz. rubber diaphragm stoppered vaccine bottles. Autoclaved at 15 lbs. for 20 minutes. Distributed with instructions to inoculate 5 ml. blood or 3 ml. bile through stopper.

Upon receipt of specimen in the laboratory, the rubber stopper was replaced with a sterile cotton plug; 0.2 ml. was streaked upon Endo and SS agar plates and incubated 22 to 24 hours at 37° C. All plates were handled similarly to that for stool cultures. Specimen was incubated 72 hours at 37° C. and streaked upon a second set of plates. The original specimen was incubated an additional 96 hours and streaked upon a third set of plates. If growth was obtained upon the first or second set of plates, organisms were identified biochemically and serologically as with stool specimens; subsequent incubation of original specimen was discontinued. If no growth was obtained from the first or second set of plates, subsequent incubation of original specimen was continued. Original specimen was autoclaved and discarded.

TABLE II. LABORATORY CONFIRMATION OF CASES

TOTAL	BLOOD CULTURES	STOOL CULTURES	AGGLUTINATION	BLOOD CULTURES AND AGGLUTINATION	BLOOD AND STOOL CULTURES	BLOOD AND STOOL CULTURES AND AGGLUTINATION	STOOL CULTURES AND AGGLUTINATION
17	0	8	0	1	0	3	5

and prior to release from the hospital or shortly thereafter. This test was also performed upon sera from both carriers and other suspected persons. The test was performed parallel upon each serum using both a live culture antigen and a Formolized antigen.* The results of all tests were negative.

Chloramphenicol (chloromycetin) was administered in doses of 250 mg. q.i.d. in all cases, except two, until the febrile stage subsided. A patient with a very severe case received 500 mg. q.i.d. during the first day, 750 mg. q.i.d. on the second, and 500 mg. q.i.d. thereafter until the febrile stage subsided upon the sixth day. In a mild case, 500 mg. was administered at three-hour intervals during the first day, 250 mg. at three-hour intervals for the next five days, and 125 mg. at three-hour intervals until the febrile stage subsided on the twentieth day. The febrile stage varied in these cases from one to twenty-three days and subsided in an average of 5.8 days after therapy was instituted. All patients received a high caloric diet accompanied by additional vitamins. Average hospitalization per patient was sixteen days, with an average post-febrile convalescence of 4.6 days (two patients with mild or subclinical cases and one with a moderate case were treated at home since good isolation facilities and care were available). The antibiotic was well tolerated in all instances, with no toxicity reported. No serious complications were recorded. At least three negative stool cultures per case, collected upon consecutive days, were obtained prior to release from the hospital or isolation. In the subsequent follow-up examinations, all patients continued to give negative stool cultures at four-week intervals for twenty weeks after release, except two. In one case which had been of a severe type, one stool culture of three submitted was positive for *S. typhosa* four weeks after release; three negative cultures were reported at the end of eight, twelve, sixteen, and twenty weeks. In a mild case with a history of adequate immunization within the previous two years, two of three stool cultures submitted were positive four weeks after release; three negative cultures were reported at the end of eight, twelve, sixteen, and twenty weeks. Follow-up of these cases will continue for at least one year. Chloromycetin therapy did not alter or modify the bacteriophage type of *S. typhosa* involved in this epidemic.

A. faecalis was isolated from stool specimens of eight of the seventeen typhoid cases during the convalescent stage. These specimens were negative for *S. typhosa* during the convalescent state. *A. faecalis* was isolated also from five suspected cases, unconfirmed for *S. typhosa*, with typhoid-like symptoms. In one instance it was isolated from a blood culture; in one case from

*Vi cultures and control serum were supplied by Salmonella Laboratory, Communicable Disease Center, Public Health Service, Chamblee, Ga., and the Army Medical Center, Washington, D. C.

Formolized Vi suspension was supplied by the Army Medical Center.

both blood and stool cultures; and from stool cultures in three cases. The symptoms of one case (an adult) were considered severe; one was moderate and three were mild or subclinical. The ages of these individuals were 8, 9, 15, 19, and 29 years. This confirms the observations of Marshall,[2] McIntyre,[3] Raeburn,[4] Vorhies and Wilen,[6] and Wyatt.[7]

SUMMARY

An epidemic of typhoid fever at Spenard, Alaska, during the months of March and April, 1950, is reported. This epidemic involved seventeen persons between the ages of 4 and 11 years, with no adult patients or deaths. The ages of these patients varied from the usual and currently reported age distribution for the disease which involves teen-age children and young adults. An adult carrier was incriminated in the epidemic.

The strain of *S. typhosa* responsible for the epidemic was bacteriophage typed as "Epidemic Strain—Untypeable Vi."

Chloramphenicol (chloromycetin) therapy accompanied by a high caloric-vitamin diet was effective in treating all cases. The febrile stage varied from 1 to 23 days and subsided in an average of 5.8 days after therapy was instituted. There was an average postfebrile convalescence of 4.6 days. Average hospitalization per case was sixteen days. The antibiotic was well tolerated by all, with no toxicity or serious complications reported.

Chloramphenicol (Chloromycetin) therapy did not clear up the carrier state. During the course of treatment, it did not alter or modify the bacteriophage type of the strain of organisms responsible for the epidemic.

The Vi agglutination test, screening test for the carrier state, was negative in all cases, both carriers and other suspected persons.

From five suspected cases, unconfirmed for *S. typhosa* and with typhoid-like symptoms, *A. faecalis* was isolated in both blood and stool cultures. This organism was also isolated from stool specimens of eight typhoid cases during the convalescent stage.

Grateful acknowledgment is expressed to the Alaska Department of Health for making available the field investigational records involved in this epidemic and compiled by their personnel; to the physicians of Anchorage, Alaska, for making available all confirmed and suspect case records; to the Salmonella Laboratory, Communicable Disease Center, Chamblee, Ga., for bacteriophage typing of cultures and certain materials; and to the Army Medical Center, Washington, D. C., for certain supplies.

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THE REACTION OF THE BONE MARROW TO HIGH OXYGEN TENSION IN NORMAL AND ANÉMIC GUINEA PIGS

ARTHUR COOPERBERG, M.D., AND KARL SINGER, M.D.
CHICAGO, ILL.

INTRODUCTION

IT IS generally assumed that the principal physiologic regulator of erythropoietic activity is the oxygen tension in the marrow. This concept is based chiefly on the well-supported evidence that in an environment with reduced oxygen tension reticulocytosis and polycythemia regularly develop. Few investigations, however, have been reported on the hematologic effects of exposure to increased oxygen concentrations and these have been contradictory. Bornstein[1] noticed a slight decrease of the hemoglobin level in dogs and monkeys under a pressure of 2 atmospheres of air and attributed this diminution to an increased plasma volume. Karsner[2] exposed rabbits to high oxygen tension for periods of three to seven days without observing any significant alterations in hemoglobin, erythrocytes, and reticulocytes or in the composition of the marrow. On the other hand, Campbell[3, 4] noted almost regularly a marked reduction of the hemoglobin and erythrocyte levels in rabbits, monkeys, and mice but not in cats during prolonged exposure to oxygen concentrations of 65 per cent. Boycott and Oakley[5] produced reticulocytopenia but very little anemia in rats by means of high oxygen tension. These authors interpreted their findings as due either to pulmonary changes interfering with the actual intake of oxygen from the alveoli or to the possibility that the exposure was capable of "raising the level of maturity at which red blood cells are discharged into the circulation rather than stop their production."

In patients with sickle cell anemia, Reinhard and co-workers[6] and Tinsley and associates[7] observed a consistant fall of the reticulocytes with increasing degree of anemia during prolonged administration of 80 per cent oxygen. Less pronounced diminution of the reticulocyte counts was noticed under similar conditions in patients with hereditary spherocytosis and with pernicious anemia under specific treatment. Whether this decreased, or even absent, output of reticulocytes in these anemic individuals reflected a temporary inhibition of red cell production, similar to that seen in an "aplastic crisis,"[8] or whether it resulted from a disturbance of the release mechanism of the juvenile erythrocytes from the marrow cannot be ascertained, since no conclusive data on the state of the marrow during these experiments were reported. A diminished delivery of reticulocytes does not necessarily indicate a concomitant decrease of erythrocytogenesis. The most convincing evidence that an agent exerts an influence

From the Department of Hematologic Research, Medical Research Institute, Michael Reese Hospital.

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on red cell production is to note its effect on the composition of the marrow. Since no in vivo studies of the marrow in animals or in patients exposed to high oxygen concentrations are available, it was considered of interest to correlate the alterations in the peripheral blood with those of the marrow in normal and anemic guinea pigs kept continuously under the influence of an oxygen tension of 70 per cent for various periods of time.

MATERIAL AND METHODS

Adult guinea pigs weighing from 400 to 600 grams were kept in individual cages (11 in. by 8½ in. by 8½ in.). A gas-tight box (height 53 in., width 61 in., depth 33 in.) was constructed similar to that described by Paine and co-workers.[9] This oxygen chamber could house fourteen cages. A large glass window on one side permitted ready observation of the animals. An oxygen tension of 70 per cent was maintained at a flow of approximately 2.2 liters per minute from commercial cylinder oxygen. By means of an additional flood valve, the oxygen concentration could be built up in about five to ten minutes. There were thus two inlets attached to two cylinders: one to maintain the gas level, and the other for rapid attainment of the desired oxygen concentration. A rubber flutter valve, similar to those commonly used in B. M. R. machines, served as an escape valve and was placed on the side of the box opposite to the inlets. The oxygen concentration was checked at frequent intervals during the day by means of a Beckman oxygen analyzer. The rubber tube attached to the analyzer was inserted through the flutter valve to the level of the cages before the determination of the oxygen tension was made. A tray of soda lime served to absorb the CO_2. Constancy of the temperature (20° C.) was maintained by means of a refrigerating unit.

The oxygen tension in the chamber was kept at 70 per cent. This level was used because of reports that concentrations greater than this usually lead to pneumonia and rapid death of the experimental animals, whereas below this level the pulmonary lesions were much less pronounced.[9]

The animals were fed lettuce and Purina rabbit chow. Enough food to last three to four days was placed in the cages, thus minimizing the exposure to atmospheric air during the time needed for cleaning the cages and replacement of food.

Blood was obtained by heart puncture from the unanesthetized animal in amounts approximating 0.3 c.c. and kept from clotting by a few crystals of an ammonium-potassium-oxalate mixture. Red cell counts were done with National Bureau of Standards certified pipettes and hemocytometers. Hemoglobin determinations were performed in a Klett-Summerson photoelectric colorimeter. Reticulocytes were counted on dry stained films, using a minimum of 2,000 red cells for their evaluation. Bone marrow aspirations were performed on alternate iliac crests and the films stained with Wright-Giemsa. Only preparations containing marrow particles were evaluated. At least 500 cells were counted to determine the percentage of the various nucleated red cells.

Usually the heart puncture and bone marrow aspiration could be performed in fifteen to thirty minutes. During this period the cages and the oxygen chamber were put in order. Thus, the exposure of an animal to atmospheric air rarely exceeded this time interval on the days when tests were performed, and this was done only every three to four days. Since the appetite of the animals diminished markedly under high oxygen tension, sufficient food was always present.

For the production of an acute hemolytic anemia in the guinea pigs an anti-red cell serum, obtained by immunizing rabbits with washed guinea pig erythrocytes, was used.[10] When the titer of the immune serum was greater than 1:256, the rabbit was bled by heart puncture and the serum stored in a deep freeze.

RESULTS

(1) Toxic Manifestations and Pulmonary Changes.—Within a few hours of exposure to 70 per cent oxygen (540 mm. Hg pressure), it was evident that

the respiratory rate of the animals had increased, and that their appetite had markedly diminished. By the fifth day there was a weight loss of 6 to 10 per cent, but on further exposure many guinea pigs became acclimatized and regained their original weight or even showed a gain in weight. Similar observations have been made by other investigators.[3, 9, 11]

Pulmonary changes were of several types and were seen in varying degrees in almost every guinea pig. Five of the animals died with massive induration and congestion of the lungs by the seventh or eighth day; in these the heart was distended and flabby. In some animals, which succumbed following a heart puncture, the lungs showed all transitions from very slight to marked congestion, edema and pneumonia affecting one or more lobes. The most constant finding was a pale, pink, distended lung which failed to collapse when removed from

TABLE I. EFFECT OF 70 PER CENT OXYGEN TENSION ON NORMAL GUINEA PIGS

GUINEA PIG	DAY	HGB. (%)	R.B.C. (MILLIONS)	RETICULOCYTES %	RETICULOCYTES TOTAL/C.MM. IN 1,000	BONE MARROW ERYTHROBLASTS (%)	REMARKS
1	0	98	4.81	3.3	161	24.4	
	9	91	4.50	0.3	11	3.4	
	16	90	4.61	0.9	42	14.4	
	24	91	4.95	3.7	184	22.4	
	36	92	4.67	2.8	131	--	
2	0	98	4.93	2.2	106	11.5	Died of pericardial hemorrhage following heart puncture. Lungs appeared normal
	9	93	4.77	0.2	10	2.2	
	16	92	5.08	0.5	25	5.2	
3	0	98	4.91	3.1	152	24.2	Died. Slight lung changes
	3	105	5.28	1.8	95	--	
	6	104	4.99	0.4	20	3.4	
4	0	99	5.08	0.7	36	11.8	
	3	94	4.95	0.3	13	--	
	6	100	4.95	0.8	40	--	
	8	93	4.62	0.3	14	1.6	
	17	82	4.32	3.4	147	30.0	
	24	90	4.50	0.9	38	16.8	
	36	87	4.54	1.2	55	--	
5	0	89	4.49	3.5	157	19.4	
	3	105	4.95	3.0	149	--	
	6	105	5.03	0.3	15	--	
	8	92	4.69	0.2	7	3.0	
	17	88	4.43	2.0	89	5.8	
	23	85	4.47	7.3	324	14.6	
	27	82	4.13	5.7	360	17.6	
6	0	89	4.40	2.3	101	20.8	
	6	103	4.96	0.2	8	--	
	8	98	4.99	0.4	18	4.0	
	17	84	4.15	0.5	29	--	
	23	86	4.26	2.7	115	13.4	
	35	83	4.20	3.3	138	--	
7	0	95	4.86	0.9	44	17.6	
	7	93	4.90	0.8	39	1.2	
	14	77	3.96	2.9	115	14.8	
	19	81	4.23	5.6	237	31.4	
	28	80	4.14	4.4	182	--	
8	0	98	4.76	1.4	71	15.4	Died of pericardial hemorrhage. Moderate lung changes
	9	87	4.47	1.1	49	10.0	
	16	86	4.61	2.3	106	19.2	
	28	84	4.29	4.7	201	17.6	

the thorax. It was usually crepitant and pitted on pressure. Occasionally, varying portions of one or more lobes were dark and firm, suggesting pneumonic infiltration.

Nine guinea pigs were killed after a stay of eight days in the oxygen chamber. Four of these had been injected with 1.0 c.c. of anti-red cell serum, and in one 6.0 c.c. of blood had been removed by heart puncture. In this group the lungs were normal in one instance, only distended and edematous in four, and in the remaining four animals there was evidence of pneumonitis.

Another group of eight guinea pigs was killed after exposure to 70 per cent oxygen tension for thirty-six to forty-eight days. In three of these animals the lungs were only slightly involved, but in the others there was some scarring and shrinkage, or small areas of induration.

(2) Hematologic Effects of High Oxygen Tension in Normal Animals.—Ten normal guinea pigs were exposed to 70 per cent oxygen tension for six to thirty-six days. Two died before any conclusive studies could be completed. Of the remaining animals, after exposure for seven to ten days, seven developed a marked erythroblastopenia in the marrow concomitant with the decrease of the reticulocyte count in the circulating blood (Table I). A slight reduction in the hemoglobin and erythrocyte values was also noticeable. In the eighth animal, the diminution of red cell production and release was less pronounced. With more prolonged exposure, the abnormal blood and marrow findings tended to return to normal. In a few instances an actual reticulocytosis occurred.

On the basis of an average reticulocyte count of 103,500 per cubic millimeter at the start of the experiment, and an average lowest figure of 19,625 reticulated erythrocytes per cubic millimeter at the height of the effect, the mean decrease in reticulocyte output for the eight animals may be computed as being 81.1 per cent. Correspondingly, at the beginning of the experiment, the average percentage of nucleated red cells in the marrow was 18.1 and at the time of the most marked depression 3.6, thus indicating an average decline in erythropoietic activity of 80.2 per cent.

TABLE II. COMPARISON OF AVERAGE COMPOSITION OF ERYTHROBLASTS AT THE BEGINNING OF EXPOSURE TO 70 PER CENT OXYGEN TENSION AND AT MOST MARKED DEPRESSION OF ERYTHROPOIESIS

TYPE OF EXPERIMENT	TOTAL PERCENTAGE ERYTHROBLASTS	PROERYTHROBLASTS	BASOPHILIC	POLYCHROMATOPHILIC	OXYPHILIC
			NORMOBLASTS		
			PER CENT OF ERYTHROBLASTS		
Before exposure	18.1	2.8	26.0	59.2	12.0
At height of depression	3.6	5.6	38.9	41.7	13.8

Table II summarizes the average composition of the erythrons before and at the height of the effect of the elevated oxygen tension. As can be seen, there was no clear-cut maturation arrest at any particular level of erythrocytogenesis. It is difficult to decide whether the differences in the percentages of the various red cell precursors are of any significance since they are not striking and are based on a relatively small number of animals.

(3) Hematologic Effects of High Oxygen Tension in Experimentally Produced Hemolytic Anemias.—Single subcutaneous injections of 0.5 and of 1.0 c.c. of anti-red cell serum were found adequate to produce anemia, reticulocytosis,

TABLE III. EFFECT OF 70 PER CENT OXYGEN TENSION IN PRESENCE OF ACUTE HEMOLYTIC ANEMIA PRODUCED BY 0.5 C.C. OF ANTI-RED CELL SERUM

GUINEA PIG	DAY	HGB. (%)	R.B.C. (MILLIONS)	RETICULOCYTES %	RETICULOCYTES TOTAL/C.MM. IN 1,000	BONE MARROW ERYTHROBLASTS %	REMARKS
				(A) Animals kept in air			
9	0	106	5.44	2.8	152	15.8	
	5	83	4.34	12.5	540	---	
	8	94	4.69	33.3	1,563	30.6	
	11	96	4.88	7.0	341	---	
	25	104	5.27	0.8	42	---	
10	0	96	4.97	2.5	124	9.0	
	5	91	4.60	11.0	505	---	
	8	93	4.69	13.3	612	29.4	
	11	97	4.99	8.4	419	---	
	25	111	5.63	1.5	85	10.2	
11	0	89	4.60	0.9	39	18.2	
	5	95	4.81	9.4	450	---	
	8	96	4.99	5.8	287	30.6	
	11	94	4.76	3.2	152	---	
12	0	95	4.89	1.7	81	11.4	
	5	84	4.15	8.5	348	---	
	8	91	4.53	13.8	623	30.5	
	11	101	5.10	3.9	200	---	
	25	107	5.50	1.7	94	---	
				(B) Animals kept under 70 per cent oxygen tension			
13	0	102	4.87	1.7	83	10.2	Died. Pericardial hemorrhage following heart puncture. No lung changes
	5	66	3.19	1.0	34	---	
	8	52	2.38	22.7	540	56.4	
14	0	89	4.49	1.8	81	---	
	8	83	4.24	9.0	382	35.4	
	12	91	4.54	11.4	518	---	
	15	78	3.98	2.5	98	5.6	
	25	67	3.47	2.7	94	17.0	
15	0	94	4.81	2.2	104	12.4	Died. Pericardial hemorrhage. Minimal lung changes
	5	84	4.43	1.4	62	11.6	
16	0	99	5.09	2.8	143	25.6	
	5	69	3.15	3.1	98	---	
	8	71	3.60	17.1	615	43.2	
	12	78	3.92	3.8	149	---	
17	0	96	4.98	2.0	100	18.8	Died on 12th day. Severe lung changes
	5	90	4.59	3.0	140	---	
	8	82	4.28	11.5	495	52.8	
18	0	90	4.58	1.2	53	8.8	
	5	84	4.43	16.9	747	---	
	8	81	4.05	16.1	652	42.0	
	13	88	4.52	11.3	511	29.6	
19	0	105	5.16	1.5	75	8.2	Died on 8th day. Pericardial hemorrhage. Severe lung changes
	5	67	3.44	8.7	301	---	
20	0	104	5.08	3.3	165	21.4	Air hunger after removal from oxygen chamber. Died. Severe lung changes
	5	90	4.51	2.7	121	---	
	7	65	3.38	2.8	95	16.4	

and erythrocytic hyperplasia of the marrow. Individual animals showed considerable variations in the degree of response to a given dosage, an observation which has been made also by other workers.[10]

(a) Effects of Small Doses of Anti-Red Cell Serum: A group of twelve guinea pigs was injected with 0.5 c.c. of the immune serum. Eight animals were placed in the oxygen chamber whereas the other four were left in air as controls. The results of these studies are summarized in Table III. A mild anemia of varying degree developed in most guinea pigs and was somewhat more severe in those exposed to high oxygen concentrations. Thus, the average hemoglobin fell from 96 to 88 per cent in the controls, and from 97 to 71 per cent in the exposed animals.

By the fifth day, a significant reticulocytosis was noted in the controls but only two of the animals in the oxygen chamber (18 and 19) showed a similar rise. The average reticulocyte count at this time was 461,000 per cubic millimeter for the controls, and 215,000 per cubic millimeter for the treated group. The maximal reticulocyte response was usually seen by the eighth day and averaged 771,000 per cubic millimeter for the controls versus 463,000 per cubic millimeter for the exposed animals.

These figures indicate that in the presence of an acute hemolytic process the reticulocyte response was definitely delayed and diminished by the high oxygen concentration, but could not be entirely prevented.

The nucleated cells in the marrow on the eighth day (maximal reticulocyte output) averaged 30 per cent in the controls and 41 per cent in the oxygen-treated animals. The average composition of the erythroblasts may be seen in Table IV. Again, no maturation arrest was noticeable. The finding of a dis-

TABLE IV. AVERAGE COMPOSITION OF THE ERYTHRONS IN GUINEA PIGS WITH ACUTE HEMOLYTIC ANEMIA ON THE DAY OF MAXIMAL RETICULOCYTE OUTPUT

TYPE OF EXPERIMENT	TOTAL PERCENTAGE ERYTHROBLASTS	PROERYTHROBLASTS	BASOPHILIC	POLYCHROMATOPHILIC	OXYPHILIC
			NORMOBLASTS		
		PER CENT OF ERYTHROBLASTS			
Animals kept in air, 0.5 c.c. of serum	30.1	4.1	29.2	61.6	5.1
Animals exposed to 70 % oxygen tension, 0.5 % c.c. of serum	41.0	3.1	18.4	77.2	1.3
Animals kept in air, 1.0 c.c. of serum	38.1	4.9	23.2	71.0	0.9
Animals exposed to 70 % oxygen tension, 1.0 c.c. of serum	39.1	3.3	22.5	67.8	6.4

tinctly decreased reticulocyte output concomitant with an increased number of nucleated red cell precursors, under the influence of the high oxygen tension, is of particular interest. This dissociation seems to suggest that the high oxygen concentration, when counteracted by the stimulus of a hemolytic process, is incapable of influencing the formation of nucleated red cells, but inhibits either the production or the release of the reticulocytes. Unfortunately, we cannot

supply any comparative data concerning reticulocyte counts in the marrows of our experimental animals, which might have been of value in further elucidating this problem.

(b) Effects of Larger Doses of Anti-Red Cell Serum: Twelve animals were injected with 1.0 c.c. of the potent serum; six guinea pigs were kept in air as controls and the others placed in the oxygen chamber. As can be seen from Table V, the results obtained with the use of the larger doses of the hemolytic

TABLE V. EFFECT OF 70 PER CENT OXYGEN TENSION IN PRESENCE OF ACUTE HEMOLYTIC ANEMIA PRODUCED BY 1.0 C.C. OF ANTI-RED CELL SERUM

GUINEA PIG	DAY	HGB. (%)	R.B.C. (MILLIONS)	RETICULOCYTES %	RETICULOCYTES TOTAL/C.MM. IN 1,000	BONE MARROW ERYTHROBLASTS (%)	REMARKS
(A) Animals kept in air							
21	0	101	5.22	0.8	42	8.4	
	5	62	3.15	49.6	1,562	---	
	8	84	4.24	39.4	1,670	35.0	
	13	105	5.05	10.4	525	27.2	
22	0	112	5.52	1.4	77	15.2	
	5	94	4.79	6.6	316	---	
	8	90	4.70	14.2	667	34.8	
	13	98	4.86	7.3	355	23.0	
23	0	95	4.78	2.4	114	24.6	
	5	36	1.91	6.9	131	---	
	6	30	1.62	41.4	672	---	
	8	43	2.28	34.1	777	58.6	
24	0	86	4.47	3.7	165	13.4	
	5	73	3.61	11.4	411	---	
	8	87	4.36	52.0	2,272	34.8	
	13	88	4.57	6.7	306	33.0	
25	0	99	5.03	2.3	115	18.0	
	5	32	1.70	23.6	402	---	
	8	66	3.44	70.5	2,425	35.2	
26	0	96	4.87	3.0	146	8.0	
	5	41	2.20	37.2	818	---	
	8	78	4.05	58.0	2,349	30.2	
	13	87	4.37	4.2	183	20.8	
(B) Animals kept under 70 per cent oxygen tension							
27	0	101	5.11	1.8	92	16.0	Killed. Lungs pale pink, distended, edema
	3	86	4.47	4.3	338	20.4	
	5	78	4.01	13.9	557	41.2	
	8	78	4.00	22.7	908	42.0	
28	0	104	5.28	0.9	48	8.8	Killed. Lungs appeared normal
	3	86	4.41	2.1	93	13.2	
	5	66	3.48	2.4	83	18.4	
	8	69	3.52	23.8	837	37.2	
29	0	94	4.82	3.6	174	28.0	Killed. Pneumonic infiltration as well as edema
	3	63	3.21	4.1	132	13.8	
	5	57	2.96	11.7	346	16.2	
	8	66	3.40	43.3	1,472	27.6	
30	0	108	5.43	3.1	168	10.2	Died on 7th day. Pneumonia right and left lower lobes
	3	65	3.43	2.7	93	11.4	
	5	30	1.45	10.6	154	24.2	
31	0	91	4.58	0.6	27	9.2	
	5	76	3.95	6.9	271	---	
	8	69	3.56	15.6	555	45.6	
	13	83	4.22	4.8	203	15.6	
32	0	99	5.03	2.7	136	10.2	
	5	61	3.10	14.6	453	---	
	8	71	3.61	65.4	2,361	43.2	
	13	85	4.52	5.8	262	11.8	

agent were qualitatively similar to those observed with the smaller quantity of serum. A more severe anemia of the same degree was noted in both groups. The average hemoglobin level of the controls decreased from 98 to 54.7, and from 99 to 60 per cent in the exposed animals. The average reticulocyte count on the fifth day was 607,000 per cubic millimeter for the controls and 311,000 per cubic millimeter for the treated guinea pigs. On the eighth day (maximal reticulocyte response) the figures were 1,676,000 and 1,226,000, respectively. The average percentage of nucleated red cells in the marrow on this day amounted to 38.1 for the controls and 39.1 for the exposed animals. The average percentages for the various types of red cell precursors may be seen in Table IV. Although the relative decrease in the reticulocyte output of the animals under high oxygen tension was again quite definite, no increase of erythropoiesis in the marrows of these guinea pigs was noticeable. The data seem again to indicate that under the stress of a hemolytic process, the reticulocytes are predominantly affected on exposure to high oxygen concentrations.

(4) Hematologic Effects of High Oxygen Tension in Anemia Caused by Acute Hemorrhage.—A group of nine guinea pigs was bled by repeated heart punctures in order to reduce the hemoglobin level by about 25 per cent. The blood volume was calculated on the basis of 70 c.c. per kilogram. Five animals were placed in the oxygen chamber, whereas four were left in air as controls. The results are tabulated in Table VI. The average hemoglobin level in the controls decreased from 102 to 80 per cent by the fifth to sixth day, with corresponding figures of 103 and 72 per cent for the oxygen-treated animals. The average highest reticulocyte output was 628,000 per cubic millimeter for the controls, and 619,200 per cubic millimeter for the exposed group. The percentage of nucleated red cells in the marrow was 42.9 for the guinea pigs kept in air and 40.5 for the animals under high oxygen tension. Thus, unlike the observations on normal animals, and on those with acute hemolytic anemia, exposure to high oxygen concentrations appears to have no inhibiting influence on erythrocytogenesis and reticulocyte output in the presence of an acute anemia caused by blood loss.

DISCUSSION

Our studies were undertaken with the intention of establishing a correlation between the changes of the erythrocyte and reticulocyte levels in the peripheral blood and erythrocytogenesis of the marrow in normal and anemic guinea pigs following exposure to high oxygen tension. No adequate in vivo studies of the marrow in animals or human beings under similar exposures are available. Our findings seem to demonstrate that the reticulocytopenia following the exposure of normal animals is caused by a marked erythrocytic hypoplasia of the marrow. However, in the acute hemolytic anemias produced by injection of anti-red cell serum, the formation of nucleated red cells was certainly not depressed in the oxygen-treated guinea pigs, although the relative reticulocyte output was significantly decreased. No influence of the high oxygen concentration could be detected on either erythrocytogenesis or reticulocyte release in the acute hemorrhagic anemias.

TABLE VI. EFFECT OF 70 PER CENT OXYGEN TENSION IN PRESENCE OF ANEMIA DUE TO BLOOD LOSS

GUINEA PIG	DAY	HGB. (%)	R.B.C. (MILLIONS)	RETICULOCYTES %	RETICULOCYTES TOTAL/C.MM. IN 1,000	BONE MARROW ERYTHROBLASTS (%)	REMARKS
(A) Animals kept in air							
33	0	101	5.05	1.0	51	8.4	Bled 6.0 c.c.
	2	77	3.96	5.2	206	---	Bled 4.0 c.c.
	4	91	4.58	10.6	486	45.8	
	5	80	4.09	17.4	712	---	
	8	89	4.57	6.2	283	---	
	13	108	5.54	2.7	150	26.4	
34	0	107	5.36	1.8	97	---	Bled 6.0 c.c.
	2	79	4.09	6.3	258	---	Bled 4.0 c.c.
	4	81	4.21	9.1	383	56.4	
	5	72	3.72	14.5	540	---	
	8	90	4.61	10.9	502	---	
	13	106	5.18	3.3	171	16.2	
35	0	101	5.07	1.9	96	13.2	Bled 6.0 c.c.
	2	74	3.83	6.1	233	---	Bled 4.0 c.c.
	6	81	4.30	16.6	713	45.4	
	8	86	4.49	16.1	720	---	
	13	103	5.06	2.4	121	13.0	
36	0	99	4.99	1.0	50	7.6	Bled 4.5 c.c.
	2	84	4.20	2.6	109	---	Bled 3.5 c.c.
	6	88	4.51	12.0	541	24.2	
	8	96	4.93	8.4	414	---	
	13	102	4.99	2.9	145	18.0	
(B) Animals kept under 70 per cent oxygen tension							
37	0	105	5.11	2.9	148	18.6	Bled 6.0 c.c.
	1	79	---	---	---	---	Bled 6.0 c.c.
	3	74	3.77	13.5	508	44.8	Bled 4.0 c.c.
	5	70	3.52	19.7	693	---	
	8	92	4.71	15.7	740	---	
	13	104	5.26	1.9	100	---	
38	0	104	5.23	1.1	58	13.6	Bled 4.5 c.c.
	1	69	---	---	---	---	Bled 5.0 c.c.
	3	74	3.84	10.4	411	47.8	Bled 4.0 c.c.
	5	66	3.23	15.2	491	---	Died of pericardial hemorrhage. Lungs appeared normal
39	0	102	5.10	2.6	133	13.4	Bled 6.5 c.c.
	2	75	3.85	8.9	343	---	Bled 1.5 c.c.
	5	73	3.78	9.5	359	27.0	
	8	85	4.35	15.6	674	---	
	13	104	5.32	3.8	202	---	
40	0	100	5.00	3.3	165	---	Bled 6.0 c.c.
	2	74	3.83	6.0	230		Bled 2.5 c.c.
	5	80	4.06	21.0	853	48.4	Died of pericardial hemorrhage. Lungs appeared normal
41	0	107	5.45	3.6	196	11.8	Bled 6.0 c.c.
	2	82	4.26	6.6	281	---	Bled 4.0 c.c.
	6	77	3.98	8.5	338	34.4	
	8	77	3.92	5.7	223	---	

Diminished erythropoiesis in normal subjects, as an effect of the exposure, was assumed to exist by several workers who noted the reduction in the number of reticulocytes in the peripheral blood.[3, 5, 7] The decreased erythrocyte production cannot be maintained, however, beyond the first ten days. After this period the blood and marrow findings either return to normal values or may even show evidence of an increased red cell formation. The only explanation we have to

offer is the inevitable development of pulmonary pathology. Although variable in degree, the lungs were affected in all experimtntal animals. These lung changes may have been sufficient to obstruct the entrance of oxygen into the circulation, thus leading to a normal or even reduced oxygen tension in the tissues. Unfortunately, determinations of the arterial oxygen tension were not feasible in our experiments. Paine and co-workers[9] found that in dogs breathing 85 to 100 per cent oxygen, the arterial oxygen saturation was occasionally depressed, and that there was some correlation to the severity of the existing lung pathology. These authors also noted that oxygen concentrations of 75 per cent resulted in pulmonary damage which was much less marked, and required a week or longer to develop.

The hematologic alterations seen in our experiments with acute hemolytic anemia appear to be quite similar to those observed by Tinsley and associates[7] in patients with hereditary spherocytosis, and in patients with pernicious anemia during treatment with liver or vitamin B_{12}, who, on exposure to high oxygen tension, also exhibited a relative reticulocytopenia. In one case of familial hemolytic jaundice "the bone marrow, aspirated in the middle and the end of the oxygen period, showed no evidence of erythroid hypoplasia."[7] Since we were unable to produce chronic hemolytic anemias in our experimental animals, we were forced to use comparative studies of guinea pigs with an acute hemolytic process, kept in air and under elevated oxygen tension, for evaluation of the marrow changes. The finding that the marrow of the exposed animals did not reveal any evidence of depressed erythrocytogenesis, although the reticulocyte output was, comparatively, markedly decreased, may indicate an inhibition of either the production or the release of the reticulated erythrocytes or a combination of both mechanisms. One might expect, on account of the relative reticulocytopenia, a more severe degree of anemia in the exposed animals. This was found, however, only in the series treated with the smaller amount of anti-red cell serum and not in the group injected with larger doses. The possibility that reticulocytes ripened more rapidly under high oxygen tension and, therefore, were discharged at a higher level of maturity must also be considered, but does not seem to be very probable. Boycott and Oakley[5] assumed such a mechanism because they observed decreased reticulocyte output without any anemia. We are more inclined to favor an existing disturbance in the production or the delivery of the young erythrocytes, although our data do not permit a clear-cut decision as to which of the mechanisms was actually involved.

Reinhard and associates[6] and Tinsley and associates[7] noticed that the effect of high oxygen concentrations was much more distinct (almost complete disappearance of the reticulocytes in the blood) in subjects with sickle cell anemia than in any other investigated hemolytic syndrome. Ham and Castle[12] suggested, as an explanation of this phenomenon, that the high oxygen tension would tend to diminish the number of sickled cells in the marrow capillaries, thus decreasing blood viscosity, and, therefore, accelerate the rate of capillary blood flow. Consequently, the marrow of patients with sickle cell anemia would be exposed not only to the direct effect of the elevated oxygen concentration but also to a greater total quantity of circulating blood. In view of the absence of any depression of erythrocytogenesis in the marrow of the exposed guinea

pigs with acute hemolytic anemia, systematic studies of the marrows of patients with the various chronic hemolytic syndromes under the influence of high oxygen might be of particular interest.

The absence of any distinct hematologic effects of the elevated oxygen tension in the animals with acute hemorrhagic anemias is noteworthy. It should be emphasized that in acute bleeding the diminution of the circulating hemoglobin takes place much more abruptly than in the hemolytic syndrome, and that the marrow reaction is also more immediate. Thus, the maximal reticulocyte response is usually seen by the fifth day in the former, and by the eighth day in the latter. Whether the complete removal of the red cells from the organism, as may occur in hemorrhage, plays any role, remains an unanswered question.

The finer mechanisms whereby oxygen influences production and release of red cells is not known. Nucleated erythrocytes require oxygen for growth, yet there is much evidence that erythropoiesis is increased in conditions with a reduced oxygen tension in the blood, and diminished when oxygen tension is raised. With the methods so far employed, the oxygen tension of the marrow seems to be normal in anemias as well as in polycythemia vera.[13, 14]

Rosin and Rachmilewitz[15] showed, by means of bone marrow cultures, that the growth of normoblasts was diminished in atmospheres of less than 20 per cent oxygen and increased with concentrations of 50 per cent. Magnussen,[16] by a different technique of marrow culture, found that adequate erythropoiesis occurred in atmospheres of 10 to 40 per cent oxygen but was arrested at 2.5 per cent and at 50 per cent or higher concentrations. Thus, low oxygen tensions were not capable of direct stimulation of the marrow, whereas concentrations above 50 per cent appeared to directly inhibit red cell formation.

Reissman[17] exposed one partner of a pair of parabiotic rats to reduced oxygen tension whereas the other could breathe ordinary air. The marrow of both partners developed an erythrocytic hyperplasia, although the oxygen tension of the blood remained normal in the animal breathing air. It was, therefore, assumed that the effect of the reduced oxygen tension was not a direct one, but was mediated through a humoral factor elicited by the hypoxemia. These observations seem to support the old theory that stimulation of erythropoiesis occurs through "erythropoietins," that is, substances which reach the marrow via the blood and are produced as a reaction of the body to hypoxemia.[18, 19] Whether these hypothetic factors may also be involved under the condition of increased oxygen tension, or whether high oxygen concentrations exert a direct toxic effect on the marrow, remains undetermined. It is well established[11, 20] that oxygen in high concentrations is toxic to a number of organs, such as the central nervous system, the lungs, liver, and kidneys, and, therefore, may be directly toxic to the marrow as well. Further investigations of the interrelationship between oxygen tensions and erythropoiesis are still necessary.

SUMMARY

The hematologic effects of exposure to high oxygen concentrations were studied in normal and in anemic guinea pigs with the intention of establishing

correlations between the changes in the peripheral blood and the erythropoietic activity of the marrow. In normal animals the reticulocytopenia following the exposure is definitely caused by a marked erythrocytic hypoplasia of the marrow. In the acute hemolytic anemias produced by injection of anti-red cell serum, however, erythropoietic activity in the marrow is not depressed under the influence of the high oxygen tensions, although the reticulocyte output, in comparison with that of controls kept in air, is markedly decreased. It is assumed that in the presence of the hemolytic process, high oxygen tension inhibits predominantly the production or the release of the reticulocytes. No influence of the elevated oxygen concentration could be detected on either erythrocyte formation or reticulocyte release in acute hemorrhagic anemias.

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LABORATORY METHODS

A DUAL SEMIMICRODISTILLATION UNIT FOR SIMULTANEOUS DISTILLATION OF FORMALDEHYDE FROM OXIDIZED AND UNOXIDIZED ALIQUOTS OF URINARY CORTICOID RESIDUES

BENJAMIN F. STIMMEL, PH.D.
SAN DIEGO, CALIF.

DISTILLATION and photometric estimation of formaldehyde following periodic acid oxidation of the C-17 side chain of the adrenal corticoids has come into widespread use as a chemical method for estimation of urinary corticoids.[1, 2] However, the necessity for concomitant formaldehyde distillation from oxidized and unoxidized aliquots of each test has made the procedure somewhat tedious, especially for large series of determinations. Furthermore, alternate distillation of oxidized and unoxidized aliquots in the same apparatus presents serious possibilities for contamination.

We have found the following dual distillation unit especially helpful in overcoming these troublesome features since it permits simultaneous distillation of oxidized and unoxidized aliquots. The unique feature of the apparatus is the use of a common hardware item (broom holder) for support of each section of glassware, thereby permitting unusual mobility of each unit during its operation.

The glass distillation unit* consists of (1) a round-bottomed flask (25 ml.) with 19/22 mm. outer joint, (2) a connecting tube with a 12/5 mm. ball and socket joint for attachment to the Liebig condenser, and (3) a Liebig condenser with an aeration tube of sufficient length to extend to the bottom of a 10 ml. graduated cylinder, sealed to its distal end. Introduction of a glass bead into the distilling flask promotes smooth distillation.

The supporting frame is made from ⅜ in. plywood (12 in. by 25 in.) seated in a 12 in. by 12 in. base. The broom holders which support the distillation unit are mounted on wood shelves (¾ in. by 1⅝ in. by 2½ in.). The distillation flasks are spaced 2½ in. apart (center to center) and 2 micro H-base gas burners† are similarly spaced and clamped on a section of a door hinge which serves as a handle. Fig. 1 shows the heating unit, *A*, attached to board when not in use. The broom holders afford sufficient support to each section of the apparatus and at the same time permit easy adjustment in a vertical plane with one hand. This is particularly important in any distillation procedure in which the distillate may accidentally be drawn back into the flask after the heating element has been removed.

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*The distillation apparatus may be obtained from Kahl Scientific Instrument Co., San Diego, Calif.

†Procurable from the Central Scientific Co., Chicago, Ill.

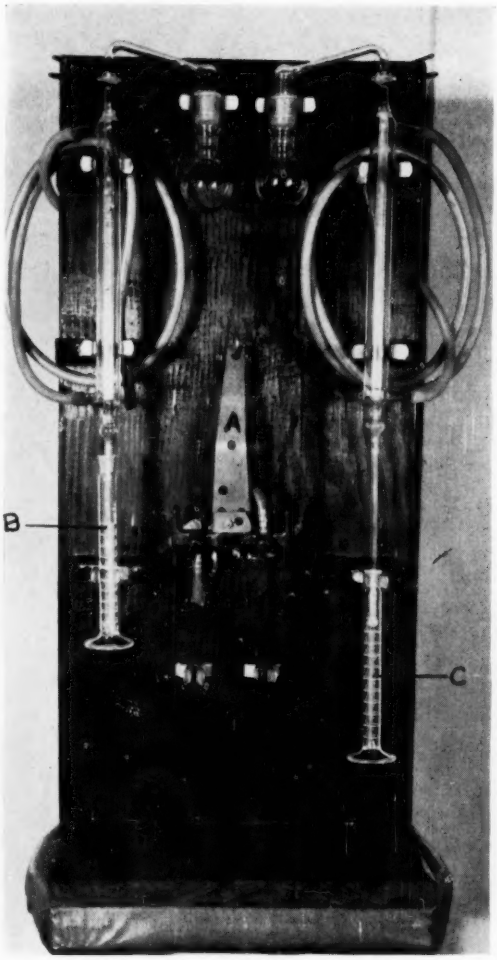

Fig. 1.

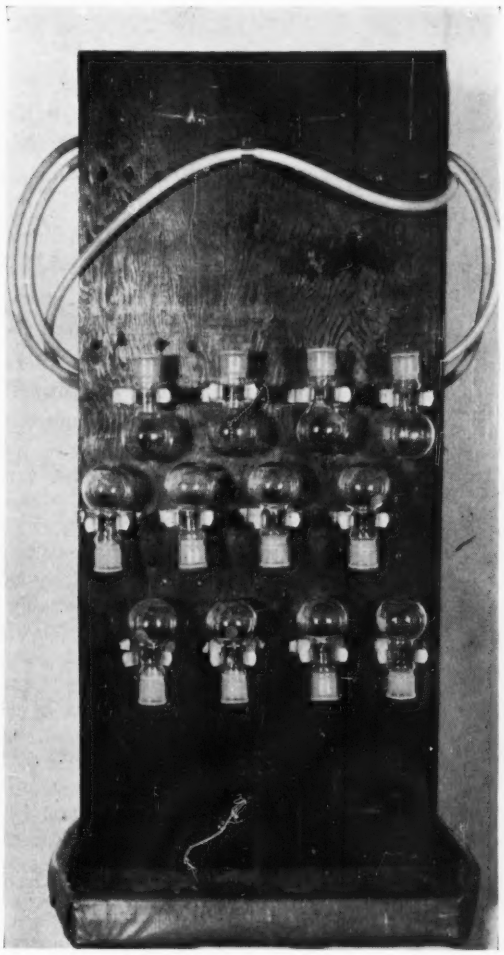

Fig. 2.

Fig. 1.—Front view of dual distillation apparatus: *A*, heating unit attached to board when not in use; *B*, position of cylinder during distillation; *C*, position of cylinder at completion of distillation.

Fig. 2.—Back view of dual distillation apparatus showing staggered position of flasks during periodic acid oxidation.

The back of the frame provides a convenient space for preparing the reaction flasks prior to distillation. The staggered spacing of flasks provides easy access with pipettes. (Fig. 2.)

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THE MICRODETERMINATION OF CHLORIDES IN SERUM AND SPINAL FLUID

VINCENT FRANCO, B.S., AND BERNARD KLEIN, PH.D.
BRONX, N. Y.

THE estimation of chloride ion in biologic fluids is an essential factor in the evaluation of the acid-base economy of the living organism. This paper describes a rapid technique which has been in daily use in our laboratory for some time, which is precise, accurate, and does not involve specialized reagents or unusual manipulation. Essentially, the procedure involves an argentimetric titration of an aliquot of the Somogyi (1945)[1] filtrate using dichlorofluorescein as absorption indicator.

MATERIALS AND METHODS

Reagents.—

Barium Hydroxide: 0.3N. Prepared from the reagent grade chemical. The solution is protected from the atmosphere by a soda lime tube.

Zinc Sulfate: 5 per cent. The solutions are adjusted so that the $ZnSO_4$ is exactly neutralized (phenolphthalien) by the $Ba(OH)_2$.[1] For use, 2 parts of the $Ba(OH)_2$ is diluted with 5 parts of chloride-free distilled water. Enough solution for a day's total requirement is prepared and kept out of contact with the atmosphere. It is reasonable to assume that the 0.086N $Ba(OH)_2$ thus prepared might be prepared directly and standardized with the $ZnSO_4$.

Sodium Chloride: 1.000 Gm. dried reagent grade NaCl is dissolved in water and diluted to 1 liter; 1 ml. = 1.0 mg.

Silver Nitrate: 0.1N.

Silver Nitrate: 0.02N. Prepared by dilution of the 0.1N solution and standardized against NaCl as described by Saifer and Kornblum.[2]

Dichlorofluorescein: 0.05 per cent in 70 per cent ethanol. Eastman Kodak No. 373 is adequate.

Procedure.—

(a) *Serum:* 7.0 ml. of the diluted $Ba(OH)_2$ solution are added to 1.0 ml. of clear serum, followed by 2.0 ml. of $ZnSO_4$ solution. The mixture is stoppered, well shaken, and centrifuged at 2,500 r.p.m. Two milliliter aliquots are transferred to a small Erlenmeyer flask, or a 6 by 1 in. test tube; 1 drop of indicator is added and the solution is titrated with 0.02N $AgNO_3$ until the first pink is seen throughout the solution.

(b) *Spinal Fluid:* 0.5 ml. of clear spinal fluid is treated with 3.5 ml. of dilute $Ba(OH)_2$ and 1.0 ml. $ZnSO_4$ solution. Two milliliter aliquots of centrifugate are titrated as already described.

Calculation.—

$$\text{mM } Cl^- \text{ per liter} = \text{ml. } 0.02N\ AgNO_3 \times F$$

$$F = \frac{5000}{58.45 \times a}$$

a = ml. 0.02N $AgNO_3$ used to titrate 1.0 mg. NaCl.

From Biochemistry Laboratory, Veterans Administration Hospital.

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RESULTS AND DISCUSSION

It had been noted in our laboratory that the original Saifer-Kornblum titration[2] gave low results. It was suspected that in the course of the alcohol-ether precipitation, some chloride had been carried down with the protein precipitate. This was in keeping with an observation made by Schales and Schales[3] who reported values 1 to 2 mM per liter higher in nondeproteinized titrations [using $Hg(NO_3)_2$-diphenylcarbazone] than in Folin-Wu filtrates. This was substantiated by comparative titrations of spinal fluid, which contains comparatively little protein, using the original alcohol-ether medium[2] and the proposed modification (Table I).

TABLE I. CHLORIDE DETERMINATION IN SPINAL FLUIDS

SAMPLE	SAIFER-KORNBLUM[2] (mM/L.)	SOMOGYI FILTRATE ALIQUOT (mM/L.)
1	127	127.5
2	127.5	128.0
3	123	123
4	131.5	131.5

We then resorted to the multiple extraction procedure as later described by Saifer, Hughes, and Weiss.[4] They offered no explanation for this variation from the original procedure although it is suspected these workers may have had the same difficulty experienced in our laboratory. A comparison of results obtained on identical specimens by this method with the original procedure as well as with the proposed technique is given in Table II. Values obtained by the original procedure were about 9 mM per liter lower, but there was excellent agreement between our method and the later Saifer modification.

TABLE II. CHLORIDE DETERMINATIONS ON 0.2 ML. SERUM; COMPARISON OF RESULTS

SAMPLE	ORIGINAL SAIFER-KORNBLUM[2] (A) (mM/L.)	MULTIPLE EXTRACTION[4] (B) (mM/L.)	DIFFERENCE (B-A) (mM/L.)	SOMOGYI FILTRATE (mM/L.)
1	94.7	102	– 7.3	
2	95.0	104.5	– 9.5	104.5
3	88.0	98.5	–10.5	98.5
4	77.0	88.5	–11.5	88.7
5	92.0	102.0	–10.0	102.5
6	89	103	–14.0	103.5
7	94	102	– 8.0	
8	79	87	– 8.0	
9	102	109	– 7.0	
		Av. diff.	– 9.9	

The extraction method is cumbersome and tedious, especially when many specimens are to be analyzed. Some time earlier, Saifer and Hughes[5] had demonstrated that satisfactory analyses could be obtained by using either the Hagedorn-Jensen[6] or Somogyi (1930)[7] zinc hydroxide filtrates. As our laboratory routinely precipitates serum proteins for the determination of glucose and urea with the Somogyi (1945)[1] $Ba(OH)_2$-$ZnSO_4$ reagents, we investigated the use of this filtrate as a medium for chloride analysis. The results obtained were eminently satisfactory. Typical results are shown in Table III.

TABLE III. COMPARISON OF CHLORIDE DETERMINATIONS BY SAIFER, HUGHES, AND WEISS[4] AND SOMOGYI FILTRATES

SPECIMEN	SAIFER, HUGHES, AND WEISS[4] (mM/L.)	SOMOGYI FILTRATE (mM/L.)	DIFFERENCE (mM/L.)
1	99.5	100.5	1.0
2	103.0	103.3	0.3
3	96.5	97.0	0.5
4	99.5	100.5	1.0
5	96.0	96.0	0
6	103.0	104.0	1.0
7	99.2	98	−1.2
8	107.0	108.0	1.0
9	106.0	106	0
10	105	106	1.0

The latest Somogyi method of deproteinization possesses many advantages over the earlier zinc methods. No additional ions are contributed to the filtrate. The reactants are chloride free and need no extensive purification (compare Sendroy[8] and Van Slyke and Hiller[9]).

No chloride ion is adsorbed by the precipitation of the proteins or by the precipitating agents. Some concern had been felt about the latter, since it is well known that $BaSO_4$ will coprecipitate Cl^-.[10] However, since the components of the precipitating agents are added in stoichiometric amounts, with no excess of Ba^{++}, the probability of coprecipitation of Cl^- is small. To test this, the precipitating agents were added to known amounts of NaCl solutions and aliquots of filtrate were analyzed as described. The results are shown in Table IV. It can be seen readily that there is little loss of Cl^-.

TABLE IV. RECOVERY OF NaCl FROM SOMOGYI FILTRATES OF PURE NaCl SOLUTIONS

SAMPLE	MG. NaCl IN SAMPLE	MG. NaCl RECOVERED	DIFFERENCE (MG.)	PER CENT RECOVERY
1	1.60	1.570	−0.03	98.1
2	1.60	1.572	−0.028	98.2
3	1.60	1.572	−0.028	98.2
4	1.60	1.569	−0.031	98.1
	Av.	1.571		98.2

Another advantage of this deproteinization is that there is no necessity for the heating period as proposed by Saifer and Hughes.[5] The proteins are precipitated immediately and no waiting period is necessary to complete the reaction other than that required to insure complete mixing of the reactants.

The method as described is remarkably reproducible. In replicate runs we were able to reproduce microtitrations of the order of 1.0 ml. to within 0.6 per cent. The titration may be carried out both in diffuse daylight or under artificial illumination. No difficulty in observing the very sharp end point was experienced. This is in contrast to the report[5] that titration of other zinc filtrates did not give distinct end points. No advantage was obtained using 1 per cent gum acacia as a protective colloid during the titration as reported recently by Stalzer, Dillon, and Vosburgh [12]

The procedure gives values in excellent agreement with a well-known, independent procedure.[3] We modified the Schales and Schales method as follows. To 2.0 ml. of Folin-Wu or Somogyi filtrate, 2.0 ml. 0.05N HNO_3 and 0.05 ml. of mixed diphenylcarbazone-bromphenol blue indicator[11] were added and the yellow solution was titrated with 0.02N $Hg(NO_3)_2$. The results are shown in Table V.

TABLE V. CHLORIDE DETERMINATION OF POOLED SERUM

SAMPLE	SCHALES AND SCHALES[3]* (mM/L.)	PROPOSED METHOD (mM/L.)	DIFFERENCE (mM/L.)
1	101.5	100.6	−0.9
2	100.5	100.6	+0.1
3	101.5	101.6	+0.1
4	101.5	101.6	+0.1
5	101.5	100.6	−0.9

*These titrations were performed with a microburette (10 ml.) graduated in 0.05 ml. divisions. It is felt that closer agreement with values found by the proposed method would have been obtained had a burette graduated in 0.001 ml. (such as was used in the proposed procedure) been used.

Here, too, the method possesses the desirable advantage of working with a single, stable, easily standardized reagent, together with the use of a stable easily prepared indicator. Recovery studies showed the method to be accurate to within 2 per cent (Table VI).

TABLE VI. RECOVERY OF NaCl FROM SOMOGYI FILTRATE OF POOLED SERUM (TO 0.5 ml. OF POOLED ANALYZED SERUM, 0.5 ml. OF NaCl SOLUTION WAS ADDED, THE PROTEINS WERE PRECIPITATED, AND THE ALIQUOTS WERE ANALYZED IN TRIPLICATE)

SAMPLE	NaCl IN SAMPLE (MG.)	NaCl ADDED	THEORETICAL	FOUND	RECOVERY (PER CENT)
1	0.202	0.5	0.702	0.712	101.4
2	0.202	0.5	0.702	0.708	100.5
3	0.202	0.5	0.702	0.715	101.8
			AV.	0.712	101.2

SUMMARY

A rapid, accurate micromethod for the estimation of chloride in blood serum and spinal fluid is described. It is based on the titration of $Ba(OH)_2$-$ZnSO_4$ filtrates with 0.02N $AgNO_3$ using dichlorofluorescein. The procedure is accurate to ±2 per cent.

We acknowledge with pleasure the technical assistance of Mr. Irving Radzin who performed numerous analyses during the development of this procedure. We are also indebted to Mr. Milton Weissman for his interest and valuable suggestions.

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A RAPID METHOD FOR THE DETERMINATION OF PARA-AMINOHIPPURIC ACID IN KIDNEY FUNCTION TESTS

CLAUS BRUN, M.D.
COPENHAGEN, DENMARK

WITH THE TECHNICAL ASSISTANCE OF BORGHILD ANGER

PARA-aminohippuric acid (PAHA) has in recent years increasingly supplanted Diodrast in kidney function tests and its suitability for this purpose is well established from theoretical as well as practical points of view.

The estimation of PAHA is usually done by diazotization methods as described e.g. by Marshall[1] and Bratton and Marshall.[2] Even if these methods are less complicated and capricious than the microiodine analyses necessary for determination of Diodrast, they have several disadvantages. The most prominent drawbacks are the lack of permanence of the developed color, instability of reagents, and interference by other substances such as traces of metals, thiocyanates, etc. The methods based on this principle have, however, the advantage of being extremely sensitive.

Simple methods for the determination of sulfonamides have been worked out based upon the yellow color which *p*-amino compounds give with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) in acid solution. Apparently they have so far not been employed for the determination of PAHA. The advantages of this principle, first worked out by Kühnau[3] (1938), Kimmig[4] (1938), and Werner[5] (1939), are simplicity, rapidity of manipulation, and permanence of color. Morris[6] studied the conditions affecting the reaction between sulfanilamide and *p*-dimethylaminobenzaldehyde in acid solution. He found a maximal development of color only within a range of pH between 1.0 and 2.0 and a very large excess of *p*-dimethylaminobenzaldehyde. He identified the compound responsible for the color as the Schiff base *p-dimethylaminobenzylidene-p-aminobenzenesulfonamide.*

As we found the method of Marshall and Bratton, which has been in use in this laboratory for some years,[7] too time consuming, we proceeded to use the benzaldehyde method and found it extremely simple and rapid. Special advantages are that precipitation of plasma proteins is unnecessary for the determination of concentrations above 5 mg. PAHA in 100 ml. plasma and that only one reagent is necessary.

In the following, details are given for the determination of PAHA in blood and urine in the two concentration ranges used in kidney function tests, i.e. 20 to 60 mg. per 100 ml. for determinations of tubular excretory mass (T_m) and 0.5 to 3 mg. per 100 ml. for clearance determinations. Of course these ranges can be altered according to the purpose with very few modifications.

From the 3rd Medical Department, Kommunehospitalet. Chief: Poul Iversen.
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TECHNIQUE

Reagents.—

(1) Para-dimethylaminobenzaldehyde (Merck or equal quality analytical grade).

(2) 2N HCl.

(3) 93 per cent (by weight) alcohol.

(4) Somogyi's zinc sulfate (12.5 Gm. of $ZnSO_4.7\ H_2O$ dissolved in 125 ml. $\frac{1}{4}N\ H_2SO_4$, distilled water to 1 L.).

(5) 0.75N NaOH.

Preparation of Reagent: Ten grams of *p*-dimethylaminobenzaldehyde are dissolved in 600 ml. 93 per cent alcohol in a 1 L. volumetric flask. Forty milliliters 2N HCl are added and distilled water to the mark. The final solution must be of white or very light yellow color.

Solutions of certain preparations of *p*-dimethylaminobenzaldehyde have a dark yellow or a greenish color which leads to high blank values. Such specimens can be purified by fractional precipitation from acid solution by the method of Adams and Coleman.[8]

Procedure.—

(1) *High ("T_m") Concentrations of PAHA in Plasma (20 to 60 mg. per 100 ml.):* Into 10 ml. of the acid alcoholic reagent in suitable test tubes 50 mm.[3] plasma are pipetted. The yellow color develops in a few seconds and is stable.

To obtain clear solutions swirl the content of the tube during pipetting in order to mix the plasma and reagent immediately. It is necessary to exert the utmost care and cleanliness in pipetting the small volume of plasma. Use a long pipette "to contain" and rinse with content of tube—or, which is easier, use a semiautomatic constriction pipette ("Carlsberg" pipette[9]).

(2) *Low ("Clearance") Concentrations of PAHA in Plasma (½ to 5 mg. per cent):* Plasma proteins are precipitated in the dilution 1:10 with $ZnSO_4$ NaOH after Somogyi. To 1 ml. plasma and 8 ml. zinc sulfate add 1 ml. NaOH; mix well and after a few minutes remove precipitate by filtration or centrifugation. Three milliliters of the clear centrifugate are added to 3 ml. *p*-dimethylaminobenzaldehyde reagent and the yellow color develops immediately.

(3) *Urine:* Urines from clearance experiments are analyzed in the same way as plasma containing "high" concentrations of PAHA. In most T_m experiments it is necessary to dilute the urine (about 1:25) before the analysis. No other procedures are needed.

(4) *Blanks:* Preinjection samples of plasma and urine treated exactly as the unknown are used. Blanks usually are low; icteric urine and plasma give only insignificantly higher blanks.

Measurement of Color.—

The color can be measured in any colorimeter against a suitable blank. We use a Lumetron photometer (model 402E) with filter M 465 (maximum transmission 4650 Å). Known standards of PAHA in the respective two ranges of

concentration give optical densities on straight lines. A 5 mm. cuvette is used for the concentrations of 20 to 60 mg. per cent, while for ½ to 5 mg. per cent a 10 mm. cuvette is chosen.

RESULTS

Recoveries of PAH from plasma and urine are shown in Table I. The mean error is 0.8 per cent.

TABLE I

	URINE	PLASMA	
PAH added until final concentration	40.0 mg. %	1.00 mg. %	25.0 mg. %
PAH found	40.3 —	0.99 —	24.8 —
Per cent	101	99	99

Influence of pH.—

With this technique the pH is 1.5 for the high concentration range and 1.8 for the lower range. The influence of pH is shown in Fig. 1.

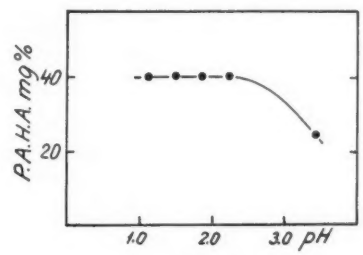

Fig. 1.

DISCUSSION

This method makes the determination of PAHA in blood and urine an extremely simple procedure and thus greatly facilitates estimations of the clearance and tubular excretory mass for PAHA. The method is, with minor modification, suitable for other ranges of concentrations as well as for the determination of other *p*-amino compounds, e.g. sulfonamides and *p*-aminosalicylic acid.

SUMMARY

An extremely simple and rapid method for the determination of *p*-aminohippuric acid in plasma and urine is described. Only one reagent is necessary and for plasma concentrations in the T_m range protein precipitation is unnecessary.

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MODIFIED GLOBIN

I. Method for Preparation From Human Erythrocytes

Max M. Strumia, M.D., and Albert B. Sample, M.S.
Bryn Mawr, Pa.

With the Technical Assistance of Margaret M. Dolan, M.T.

PREVIOUS publications[1-3] from this laboratory have considered some of the properties and clinical uses of modified globin from human erythrocytes. A limited amount of information on the method of preparation and properties of this material has been recorded[4-16] but not in the generally available literature. Since considerable interest in the clinical use of modified globin has been manifest, it seems desirable at this time to describe a detailed procedure for its preparation, with the hope of encouraging more extensive studies to evaluate its usefulness in various clinical conditions.

Basically, the procedure to be described involves the following steps:

(1) Preparation of hemoglobin solution from erythrocytes.

(2) Preparation of globin hydrochloride from hemoglobin solution.

(3) Hydrolysis of globin hydrochloride with alkali to obtain a water-soluble modified globin.

(4) Removal of impurities from modified globin by means of dialysis and adsorption.

(5) Sterilization and distribution of modified globin solution.

Satisfactory lots of modified globin have been prepared by this procedure on both laboratory and semi-pilot plant scales, which yield around 50 Gm. and 700 Gm. respectively of finished modified globin (dry basis). The experience of two pharmaceutical manufacturing concerns* with small scale production of this material has shown that it is feasible to prepare it in large quantities.

Since most of our experience with the production of modified globin has been with lots of about 700 Gm. prepared from 4 liters of packed erythrocytes (hematocrit 80 to 85 per cent), the following method will specify materials and equipment for lots of this size. This technique was adopted with minor modifications from that used for preliminary laboratory scale studies, and it should be readily adaptable to larger production with minor modifications, which, if they are not obvious, will be pointed out as permissible for more efficient handling.

From the Laboratory of Clinical Pathology and the John S. Sharpe Research Foundation, Bryn Mawr Hospital.

This paper is the twenty-third of a series on blood, plasma, and plasma substitutes from the Laboratory of Clinical Pathology and The John S. Sharpe Research Foundation of the Bryn Mawr Hospital. The work described here was aided by a grant from The John S. Sharpe Research Foundation and was in part carried out under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Bryn Mawr Hospital and a contract with the Army Medical Research and Development Board.

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*We are indebted to Smith, Kline & French Laboratories and to Sharp & Dohme, Inc., for considerable assistance in developing the technique for preparation of modified globin and for making this material available for clinical studies. Sharp & Dohme, Inc., Glenolden, Pa., now has this material available for experimental studies and is planning to prepare it on a commercial basis.

METHOD

Since it is necessary to observe extreme precautions to exclude pyrogens throughout the entire procedure for preparing modified globin, the technique which we have found satisfactory for rendering equipment pyrogen-free will be described in some detail.

Technique for Excluding Pyrogens.—

Materials:

Dichromate-sulfuric acid cleaning solution.

Pyrogen-free distilled water: Distilled water from most laboratory and production type of stills is pyrogen-free when collected if the still boiler is thoroughly cleaned at frequent intervals and if the water which distills during the first thirty minutes of each run is discarded before collection is started. Unless promptly sterilized, distilled water will not remain pyrogen-free after storage at room temperature for more than four hours or at 4° C. for more than sixteen hours.

Procedure:

Glassware: All glassware should be treated with dichromate-sulfuric acid cleaning solution, rinsed thoroughly with tap water, rinsed several times with pyrogen-free distilled water, and inverted to drain. This equipment should be used within four hours after treatment, if stored at room temperature. If oven dried immediately after cleaning and protected from dust, this glassware may be stored indefinitely before use.

Metal equipment: Prepare the same as glassware, but scrub with Alconox or other suitable detergent instead of treating with cleaning solution.

Cloth and rubber materials: Scrub with Alconox or other suitable detergent solution, rinse thoroughly with tap water, boil for five minutes in pyrogen-free distilled water, and then rinse several times in pyrogen-free distilled water. Storage conditions are the same as already described for glassware.

Preparation of Hemoglobin Solution.—

Materials:

Human erythrocytes: Sterile erythrocytes from any or all types of human blood, containing one of the usual citrate anticoagulants, are stored in the original donor bottles after removal of plasma. Erythrocytes may be stored in a refrigerator at 4° C. if they are to be used within a few days, but, if longer storage is necessary, it is advisable to freeze and store them at a temperature below −15° C. Bottles break less frequently if they are only three-fourths filled and inclined at an angle of about 15 degrees during freezing. Frozen erythrocytes should be placed in a refrigerator at 4° C. on the day before they are to be used and allowed to thaw overnight.

Sodium citrate dihydrate, U.S.P. XIV.

Equipment: Bottle, 9 liter, glass, with approximate graduations for 4 liters and 8 liters.

Cheesecloth, absorbent, Curity grade 60.

Funnel, 15 cm. diameter, with 15 cm. stem, glass.

Graduated cylinder, 1 liter.

Refrigerated room, 4° C.

Procedure: In order to minimize bacterial contamination, the process for preparation of the hemoglobin solution should be carried out in a refrigerated room at 4° C., immediately before the solution is to be treated with acid acetone.

Pour the erythrocytes from the donor bottles through one thickness of cheesecloth supported on a 15 cm. funnel which empties into the graduated 9 liter bottle. Collect approximately 5 liters of the pooled erythrocytes which have been filtered free of fibrin precipitates and clots, and mix.

Remove a 5 ml. sample of the pooled erythrocytes and have it analyzed immediately for hemoglobin content.

From the result of the hemoglobin analysis, calculate the volume of erythrocytes needed to give a 14 per cent w/v solution of hemoglobin when diluted to 8 liters, and,

using the 4 liter graduation and a 1 liter graduated cylinder, measure this volume of erythrocytes into the graduated bottle.

Dissolve 10 Gm. of sodium citrate dihydrate in 3 liters of pyrogen-free distilled water at 4° C., add this to the erythrocytes, and then add pyrogen-free distilled water to the 8 liter mark on the bottle.

Mix the solution by vigorous shaking, and store it in the refrigerated room until final arrangements are made for the acid-acetone precipitation. This solution should not be stored for more than an hour or two before use.

Preparation of Globin Hydrochloride.—

Materials:

Acetone: Reagent grade acetone may be used as received or after recovery by a single distillation, if it has been used in the following procedure for acid-acetone precipitation of globin. For each 5 gallons of acetone to be recovered, 20 Gm. of sodium hydroxide dissolved in 80 ml. of water should be placed in the still to neutralize excess acid from the globin precipitation.

Recovered acetone should meet reagent grade specifications for acidity and specific gravity.

Hemoglobin solution, 14 per cent w/v, prepared as already described.

Hydrochloric acid, 38 per cent w/w, sp. gr. 1.19, reagent grade.

Sodium hydroxide, technical flake, for acetone recovery only.

Equipment: Basket centrifuge, 43 cm., 1,700 r.p.m., with perforated stainless steel basket and removable canvas liners.

Cheesecloth, absorbent, Curity grade 60.

Drum drier: A stainless steel drum with electrical rotating device, a centrally located cylindrical screen (5 mesh) of about one-third the diameter and of the same length as the drum, and circular openings of slightly larger diameter than the screen at both ends. In use, the end openings are covered with cloth, and an electric fan is allowed to blow air through the cloth-covered ends while the drum is rotating.

Drying room: A room approximately 2.5 meters by 3.0 meters with low ceiling for easy cleaning, sterilamps, and a table approximately 0.6 by 3.0 meters with a stainless steel top.

Electric fan, 25.4 cm., nonoscillating.

Funnel, 15 cm. diameter with 15 cm. stem, glass.

Glass-lined tank, 120 liter, with a glass-coated electrically operated stirrer and an outlet valve at the bottom.

Jars, 15 cm. diameter by 45 cm. high, glass.

Rubber stopper, No. 8, with glass and rubber tubing for hemoglobin bottle.

Procedure: To 80 liters of acetone in the 120 liter glass-lined tank add 400 ml. of 38 per cent w/w hydrochloric acid, and mix.

While mixing the acid acetone so that a good vortex is formed, add hemoglobin solution at the rate of about 1 liter every five minutes, filtering it as it comes from the bottle through one layer of cheesecloth supported on a glass funnel. The stream of hemoglobin solution from the funnel should be directed into the vortex of the acid acetone so that the most rapid possible mixing is obtained.

After all of the hemoglobin has been added, the suspension of precipitated globin should be stirred for an additional thirty minutes.

With continued stirring, run the suspension from the bottom outlet of the precipitation tank into the basket centrifuge, which is lined with a canvas bag. The centrifuge should be run at about half speed (850 r.p.m.) and the suspension introduced into the bag at about the same rate as the waste acetone solution is being discharged from the drain of the centrifuge. When all of the precipitated globin has been collected in the centrifuge, spray the globin cake with acetone while still spinning until it is free of hematin as indicated by clean effluent liquid, and then wash it with an additional 10 liters of acetone.

When the wash acetone has stopped flowing from the centrifuge drain, set the centrifuge for maximum speed (1,700 r.p.m.) and allow it to run for twenty minutes to partially dry the globin cake.

Remove the canvas bag with the cake from the centrifuge to the drying room. Place the globin cake on the stainless steel table and pulverize it with a large spatula.

Place the acetone-moist powder in the screen of the drum drier, and, after covering the ends of the drier with cloth, place it on the rotating device to rotate with the fan blowing through it overnight.

Transfer the dry powder from the drum drier to a 15 by 45 cm. tared glass jar and weigh. Remove a 25 Gm. reference sample, and store the large jar of powder at 4° C. until time for it to be treated with alkali. Unless the moisture content of this powder is below 5 per cent, it should not be stored for more than a few days before use.

Determine the moisture in the globin hydrochloride by determining the weight loss on a 1 Gm. portion from the reference sample after drying for just one hour at 105° C. These drying conditions give a moisture value comparable to that obtained by desiccating. Prolonged oven drying removes hydrochloric acid as well as moisture.

From the moisture figure, calculate to a dry basis the amount of powder remaining in the large jar.

Alkaline Hydrolysis.—

Materials: Globin hydrochloride, prepared as previously described.

Hydrochloric acid, 10 per cent w/v.

Sodium hydroxide, saturated solution, carbonate free, standardized.

Water, 37° C.: Sterile, pyrogen-free distilled water heated to 37° C. Conveniently handled in 20 liter glass bottles with rubber stoppers.

Equipment: Bottles, 20 liter, glass.

Cheesecloth, absorbent, Curity grade 60.

Funnel, 15 cm. diameter with 15 cm. stem, glass.

Incubator, 37° C.

Jar, 40 liter: Glass jar, 30 cm. in diameter and 60 cm. deep, with a hole for a No. 8 rubber stopper drilled in the bottom. The jar is used on a bench having an opening in the top to accommodate the drainage outlet, which consists of a piece of 10 mm. glass tubing through the rubber stopper, with a piece of rubber tubing and a pinch clamp for a closure.

Mixer: An electric mixer with a 75 cm. stainless steel stirrer having a 7.5 cm. two-bladed propeller, a $\frac{1}{30}$ H.P. motor with a rheostat, and a suitable support.

pH Meter, Beckman model G, with external electrodes.

Refrigerated room, 4° C.

Stoppers, rubber, No. 11½.

Procedure: Into the 40 liter glass jar having a bottom outlet, measure a volume of pyrogen-free distilled water at 37° C. to give 95 ml. of water for each 4.55 Gm. of dry globin hydrochloride powder to be treated.

Add to the water a volume of carbonate-free saturated sodium hydroxide solution equivalent to 1.05 Gm. of sodium hydroxide for each 4.55 Gm. of dry globin hydrochloride powder to be treated.

Mix at a speed which gives a good vortex in the solution, and pour the powder, rather slowly, into the vortex. Stir for four minutes after all of the powder has been wet, and then turn off the mixer and allow the solution to stand for fifteen minutes. Again stir for four minutes, and allow to stand for fifteen minutes.

Drain the solution from the bottom outlet of the jar through four layers of cheesecloth supported on a 15 cm. glass funnel, and collect the filtrate in two approximately equal portions in 20 liter bottles. Place the bottles in an incubator at 37° C. for exactly twenty-seven hours.

After the incubation period, remove the bottles to a refrigerated room at 4° C., and transfer the solution to a 40 liter glass jar which has a bottom outlet. Without delay, while mixing the solution vigorously, add 10 per cent w/v hydrochloric acid to bring the pH to 7.1, using the external electrodes of the pH meter directly in the solution. This pH for globin solution at around 36° C. gives a pH of about 7.4 at 25° C.

Dialysis and Adsorption.—

Materials:

Decalso: An adsorbent available from the Permutit Company.* Before use, mix vigorously with ten times its weight of 0.5 per cent w/v anhydrous sodium carbonate, and then wash by decantation with pyrogen-free distilled water until the washings are neutral to litmus.

Distilled water, 4° C.: Pyrogen-free distilled water cooled to 4° C. and flowing at a rate of 40 liters per hour.

Modified globin solution: Solution of neutralized alkali-treated globin prepared as described.

Equipment:

Dialysis bags: Cut 75 cm. lengths of Visking sausage casing (7 cm. inflated diameter) and boil, shortly before use, in pyrogen-free distilled water for five minutes. Wash the casing thoroughly in pyrogen-free distilled water, and then twist and tie one end of each length with string, so that a water-tight seal is formed. Tie a 10 cm. length of 20 mm. glass tubing in the other end of each length of casing to serve as a spout for filling and emptying.

Dialysis jar, 120 liter, stoneware.

Refrigerated room, 4° C.

Procedure: The following procedure should be carried out entirely in a refrigerated room at 4° C.

Fill the dialysis bags about three-fourths full of the neutralized globin solution from the bottom outlet of the neutralization jar, stopper the bags with rubber stoppers, and suspend them in the dialysis jar so that the level of the globin solution in the bags is about 7.5 cm. above the level of the dialysis water. Allow the dialysis to proceed for twenty to twenty-four hours, with distilled water flowing into the bottom of the jar and overflowing from the top at a rate of about 40 liters per hour.

After the dialysis period, pour the solution from the dialysis bags into a 40 liter glass jar. Add 30 Gm. of Decalso for each liter of solution, and mix vigorously so that the Decalso is completely suspended for one hour.

After the mixing period, allow the solution to stand without mixing until the Decalso has settled. Siphon the supernatant solution into a 30 liter bottle and keep at 4° C. While final preparations are being made for the following sterilization and distribution process, analyze this solution for protein (biuret method), sodium chloride, and pH.

The protein may be adjusted at this point to any convenient concentration by dilution with water. Most of our clinical studies have been carried out with solutions containing about 4 to 4.5 per cent of globin.

If a solution isotonic with salt is desired, the additional salt may be added at this point in the form of a concentrated sodium chloride solution or by dissolving the calculated amount of sodium chloride in the water, if any, to be used for dilution. In some cases it is desirable to leave the salt concentration low. If such material is used intravenously, it must be made isotonic by the addition of dextrose to make a 5 per cent solution. When the globin concentration is adjusted to 4 to 4.5 per cent and no salt is added, the sodium chloride concentration is usually 0.1 to 0.2 per cent after dialysis and adsorption.

The pH of this solution, after the addition of water and/or salt, should be adjusted to 7.45 at 25° C.

This solution should not be stored for more than about an hour before sterilization.

Sterilization and Distribution.—

Materials:

Modified globin solution: Solution of modified globin after adsorption prepared as described.

Sodium carbonate, 0.5 per cent w/v: Prepared from reagent grade anhydrous sodium carbonate and pyrogen-free distilled water.

Sodium chloride, 0.9 per cent w/v: Prepared from reagent grade sodium chloride and pyrogen-free distilled water.

*300 West 42nd Street, New York 18, N. Y.

Equipment: Bottle caps, paper, to cover necks of 400 ml. plasma bottles.

Bottles, 9 liter and 20 liter, glass.

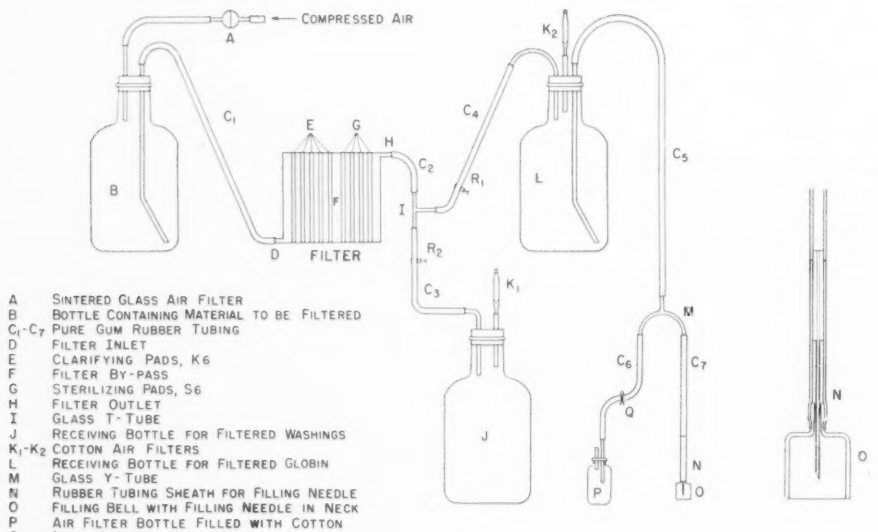
Filling bells, for 400 ml. plasma bottles, glass.

Hypodermic needles, No. 19.

Plasma bottles, 400 ml.: Treat 400 ml. plasma bottles with dichromate-sulfuric acid cleaning solution, and then rinse thoroughly with tap water and finally with pyrogen-free distilled water. Stopper and evacuate the bottles by piercing the stoppers with a No. 19 hypodermic needle which is connected to a vacuum supply having a vacuum equivalent to 73.66 cm. (29 in.) of water. Place paper caps over the stoppers and tie them with string. Sterilize these bottles in an autoclave for one-half hour at 120° C. within two hours from the time of the final rinsing.

Republic filter, model 200.

Republic filter pads, K-6 clarifying and S-6 sterilizing.



A SINTERED GLASS AIR FILTER
B BOTTLE CONTAINING MATERIAL TO BE FILTERED
C_1-C_7 PURE GUM RUBBER TUBING
D FILTER INLET
E CLARIFYING PADS, K6
F FILTER BY-PASS
G STERILIZING PADS, S6
H FILTER OUTLET
I GLASS T-TUBE
J RECEIVING BOTTLE FOR FILTERED WASHINGS
K_1-K_2 COTTON AIR FILTERS
L RECEIVING BOTTLE FOR FILTERED GLOBIN
M GLASS Y-TUBE
N RUBBER TUBING SHEATH FOR FILLING NEEDLE
O FILLING BELL WITH FILLING NEEDLE IN NECK
P AIR FILTER BOTTLE FILLED WITH COTTON
Q PINCH CLAMP
R_1-R_2 SCREW CLAMPS

FILTRATION APPARATUS

Fig. 1.

Stoppers for sterile bottles: Clean hooded rubber stoppers by boiling them in 0.5 per cent w/v hydrochloric acid for five minutes; rinse with tap water; rinse with pyrogen-free distilled water; and then boil in pyrogen-free distilled water for five minutes. These stoppers should be used immediately after cleaning.

Procedure: Set up the Republic filter as described in its operating instructions for double filtration, using K-6 clarifying pads (*E*, Fig. 1) on the inlet side of the by-pass (*F*) and S-6 sterilizing pads (*G*) on the product side. Wash the assembled filter with pads by passing through 9 liters of cold pyrogen-free 0.5 per cent w/v sodium carbonate solution and then 9 liters of cold 0.9 per cent w/v sodium chloride solution. Use a positive pressure of not over 7 pounds, and filter the air supply through a sintered glass air filter (*A*).

Thoroughly clean all parts and set up filtration apparatus as shown in Fig. 1. Autoclave the assembled apparatus for thirty minutes at 120° C. Cool to room temperature, using the cold room if it is necessary to save time.

When the filter is to be used for modified globin solution, leave screw clamp R_1 closed and screw clamp R_2 open, pass 0.9 per cent w/v sodium chloride solution, which has been cooled to 4° C., through the filter, and receive the filtrate in bottle *J* until the filtrate starts to come through colorless.

Filter globin solution with the same clamp settings as above until the first colored filtrate indicates the presence of globin solution in bottle *J*, then close clamp R_2 and open clamp R_1 so that the filtered modified globin solution is collected in bottle *L*.

After the receiving bottle *L* is half full, the filling of the individual final containers may be started. Pass the filling tube C_3 with its attachments through a small opening into the sterile filling room. All persons working in the filling room should wear sterile gowns, caps, and masks.

In the filling process, remove the paper bottle cap while flaming the mouth of the filling bell (*O*) for a few seconds, and immediately cover the neck of the bottle with the bell and jab the filling needle through the rubber stopper so that the tip of the needle is inside the bottle. The negative pressure causes the solution to flow from the receiving bottle *L* of the filtration apparatus into the plasma bottle.

Fill the bottles to contain approximately 300 ml. by comparing the depth of solution in the bottle being filled with the depth of 300 ml. of water or other liquid in a similar bottle. After the bottle has been filled, the excess vacuum is released by pinching the solution line C_3 with the fingers and opening the pinch clamp *Q* leading to the air filter *P*, allowing sterile filtered air to enter the plasma bottle. Bottles should be filled in rapid succession, flaming the filling bell between each to minimize chances for bacterial contamination.

PRODUCT CONTROL

At the beginning, the middle, and the end of each series of bottles that is filled, duplicate cultures for sterility tests on globin solution from the filling system are taken in tryptose broth and in sodium thioglycollate broth contained in evacuated 100 ml. culture bottles. Use the same filling procedure that is used for the plasma bottles, but collect only about 2 ml. of globin for each culture. For the tryptose broth cultures, the excess vacuum is removed in the same manner as for plasma bottles, but the excess vacuum is not removed from the sodium thioglycollate bottles.

Three of the 400 ml. bottles of globin solution should be tested for sterility, toxicity, and pyrogenicity. Sterility tests are carried out according to the standard methods of the National Institute of Health.[17] The directions for pyrogen testing contained in the *Pharmacopoeia of the United States*[18] are followed with the following modifications: Not one of three normal rabbits receiving 400 mg. per kilogram of modified globin contained in 8 to 15 ml. of solution show a temperature rise above 0.5° C. If only one of the three rabbits shows a greater temperature rise, the material may be retested in two additional rabbits, both of which must not have temperature rises above 0.5° C.

One bottle of globin from each lot should be analyzed for globin concentration by means of a Kjeldahl nitrogen determination, for sodium chloride, and for the "heat cloud point." The "heat cloud point," which is the temperature at which the first visible cloud appears on slowly heating modified globin solutions in a water bath with continuous stirring, should be at least 50° C. for material prepared by the standard method after adjusting the pH to exactly 7.4. It is used in controlling the length of time for alkali treatment, as well as for a safety control for the finished product.

The following tolerances have been established for the results of the various analyses of modified globin solutions:

Total nitrogen	6.67 to 7.50 mg./ml.
Modified globin (calculated from nitrogen on the basis of 16.67 per cent N)	4.0 to 4.5%w/v
pH at 25° C.	7.3 to 7.6
Heat cloud point (pH 7.40)	50° to 70° C.
Sodium chloride (except for low salt lots)	0.8% to 1.0%w/v

As a check for spotty contamination, all bottles of globin solution should be stored at room temperature for two weeks and then at 37° C. for two weeks, being carefully observed after each period for visible evidence of bacterial growth. It also appears that these incubation periods serve the additional purpose of eliminating a slight toxicity of undetermined nature which has occurred in some lots of globin prepared without this treatment.

To determine safety in animals, two mice are injected intraperitoneally with 0.5 ml. of a 4 per cent w/v solution, or equivalent, of modified globin and observed for five days for symptoms of toxic reactions.

In human testing, 400 to 500 ml. of a 4 per cent w/v solution of modified globin should be given intravenously at the rate of about 3 mg. per kilogram per minute (about 5 ml. of a 4 per cent solution per minute for a patient weighing 70 kilograms) and the patient observed with hourly temperature readings for a period of five hours.

If all of the foregoing safety tests are passed by a lot of globin solution, it may be stored for parenteral use in human beings for more than two years at room temperature, or indefinitely if dried from the frozen state.

DISCUSSION

It is rather obvious that the extreme precautions necessary for exclusion of bacteria and pyrogens from any parenteral solution should be observed throughout the procedure for preparing modified globin, but it should be emphasized that the extent of alkali treatment of globin is the most critical part of the production process. Although, with a rigorously standardized technique, the recommended twenty-seven hour treatment with alkali should produce satisfactory modified globin by the method described, it is possible that under some conditions the time of this treatment might need adjustment. If so, the isoelectric point of the globin should be determined at intervals before completion of the alkali treatment, to determine the optimum time.

The isoelectric point may be determined by extremely careful titration of a 10 ml. portion of alkali-treated globin with 10% w/v hydrochloric acid until the first visible cloud appears. This point should be between pH 5.5 and 6.5. If the cloud point is above this range, longer treatment is required, but if below this range, the globin has been overtreated. Overtreated globin may contain undesirable low molecular weight materials, and, if undertreated, it may precipitate in the blood when administered intravenously.

A "heat cloud point" on the finished product between 50 and 70° C. indicates that the isoelectric point is within the desired range; however, if adjustment of the treatment time is necessary, it is advisable to use both control tests in establishing it. Once the time for alkali treatment has been determined, a carefully standardized technique for the production procedure will assure uniform lots of modified globin.

If it is desirable to have pure globin, residual plasma and leucocytes may be removed from the erythrocytes by three washes with equal volumes of 0.9 per cent w/v sodium chloride solution, by mixing the erythrocytes with the salt solution, centrifuging to separate the layers, and aspirating the wash solution and leucocytes. For general clinical use, however, globin prepared from erythrocytes containing leucocytes and a small amount of plasma is perfectly satisfactory.

At one stage in the development of the production process, it seemed desirable to limit the range of molecular species present in the modified globin, and a sodium chloride precipitation in acid solution was used to eliminate most of the low molecular weight material. This added step was used with success in the production procedure for some time, but it has now been eliminated, since, for general clinical use, globin prepared without it is quite satisfactory.

Dialysis serves the purpose of removing low molecular weight protein decomposition products formed during alkali treatment as well as excess sodium chloride.

A number of satisfactory lots of globin have been prepared without the adsorption with Decalso, but it appears that we have obtained a lower incidence of human vasomotor reactions since this technique was added to the standard procedure. It is possible that further studies will show that it would be reasonable to eliminate this step.

The vasomotor reactions noted after the administration of globin are very similar to those observed after administration of hydrolysates commonly used as a source of nitrogen feeding. These reactions have been mild and have not caused serious concern since they may be readily controlled by decreasing the rate of administration of the globin solution.

The yield of modified globin has been between 60 and 70 per cent of the hemoglobin.

Modified globins have been prepared by this method from the erythrocytes of various animals, but detailed studies of these materials have not been completed.

SUMMARY

A detailed procedure is presented for the preparation of an alkali-modified human globin suitable for parenteral use in various clinical conditions. Erythrocytes, which are most often discarded after blood plasma processing, are utilized.

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A CONTINUOUS-RECORDING AUTOMATIC BUBBLE FLOW METER

A. Sokalchuk, M.D., Arnold S. J. Lee, A.B., and Keith F. Killam, B.S.
Philadelphia, Pa.

IN 1934 Soskin[1] introduced the concept of measuring mean rate of blood flow by visually timing the transit of an air bubble injected into a system containing a length of glass tubing of known volume. The reliability and simplicity of the bubble flow meter principle stimulated a number of improvements and applications,[2-6] including coronary inflow measurements.

Photoelectric timing of the bubble transit time employed by Selkurt[5] improved the accuracy of the method and facilitated more frequent observations. Lu[7] achieved continuous recording in isolated perfused hearts by adapting photoelectric timing of an opaque drop to control a modified Ludwig-type Stromuhr. Rubber diaphragms separated the perfusate from a distilled water system containing the opaque drop. The present report is concerned with the details of a continuous-recording automatic bubble flow meter which was designed by our laboratories and adapted to recording coronary blood inflow in the anesthetized animal. However, the unique design of this new automatic valve switching device makes its utility obvious in other fields of flow measurement.

PRINCIPLE

The impulse derived from the photoelectric timing of an introduced air bubble is utilized to activate a specially designed disk valve. The valve action selects in alternation one pair of the two branches into which both input and output tubes are divided and thus directs the forward motion of blood so as to oscillate the air bubble in a tube of known volume between two photocells. Continuous automatic recording is thus achieved.

Mechanical Details.—An isometric view of the special disk valve and tubing assembly and the relationship of light source, photocells, and valve switching solenoids are shown in Figs. 1 and 2. Arterial blood supplied by input tube *I* is distributed to tubes *A* and *B*. The outflow tube *O* collects blood from tubes *M* and *N* and supplies blood to the coronary artery. The relative position of the mobile disk *VM* to the stationary disk *VS* of the special disk valve determines the direction of blood flow in tube *T*. An air bubble inserted into tube *I* is carried to the loop of tube *T*. The two photocells P_1 and P_2 (timing stations) guarding ports *C* and *D* of disk *VS* will permit the bubble to enter the loop but will not allow its passage beyond them toward the valve. Once the bubble has entered the loop, the photocells cause it to oscillate between them by activating valve-switching solenoids, *S*.

Friction is minimized and leakage of blood prevented at the contacting surfaces of the disk valve by the use of an appropriate lubricant, such as a mixture of ⅓ white petrolatum and ⅔ liquid petrolatum, and proper spring tension. An air trap is connected to tube *O* to prevent air from entering the circulatory bed. The air outlet tube from the trap is also used

From the Research Division, Smith, Kline & French Laboratories.
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to collect blood during calibration of the device. A shunt, normally closed, is placed beyond the air trap and between tubes *I* and *O*. Coronary blood flow is retained during calibration by opening the shunt and closing tube *O* between the air trap and the shunt.

To minimize the heating of blood, the light source *L*, a Burton microscope lamp, is fitted with a cooling filter and the light conducted in bent Lucite tubes, L_1, to the timing sites of the nonwettable plastic tube *T*. At this location the tube *T* is shielded with a slot so as to permit only transmitted light to reach the photocell.

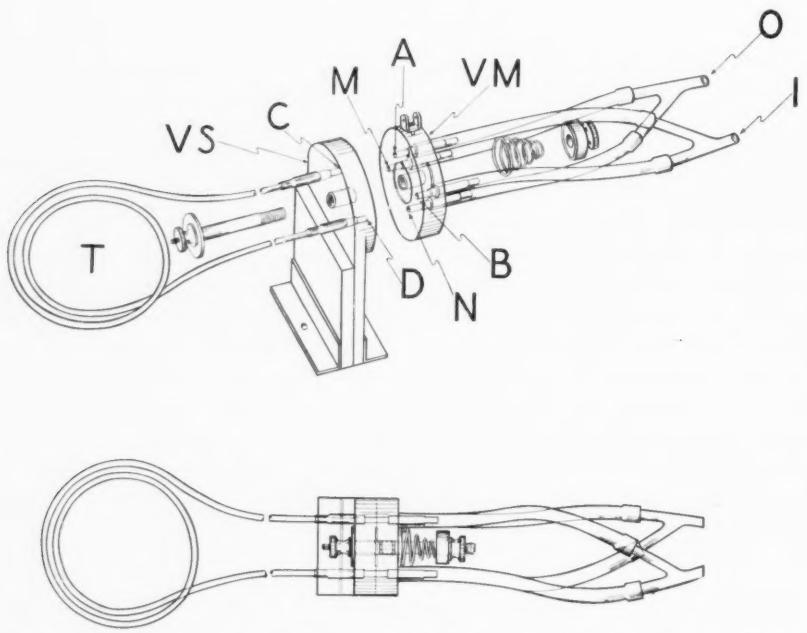

Fig. 1.—Isometric view of special disk valve and tubing circuit. *I*, input tube; *O*, outflow tube; *VM*, mobile disk to which are attached the ends of outflow tube *O* at *M* and *N* and input tube *I* at *A* and *B*; *VS*, stationary disk; *T*, flow tube loop; *C* and *D*, guard ports to flow tube loop *T*. See text for details of operation.

Electrical Details.—The electronic circuit which controls the valve-switching solenoids and provides signal magnet recording is shown in Fig. 3. Each phototube, after a single stage of amplification, excites a conventional thyratron relay which energizes the valve-switching solenoid with a minimum of disturbance to other sensitive laboratory apparatus. Sensitivity controls are placed in the amplifier-thyratron coupling circuits. At the beginning of each experiment these controls are adjusted for maximum efficiency. Bias for the thyratrons is provided by a 10 volt winding on the filament transformer. The phase of this transformer with respect to the main power transformer must be such that when the plate of the thyratron is positive, the grid is negative.

To prevent contamination by blood and other fluids, the electronic circuit is constructed as a separate unit and connected to the mechanical unit (Fig. 2) with plugged extension cords to receptacles *1*, *2*, and *3*.

Method of Recording.—Our present model is designed to operate a simple signal magnet. This permits recording on kymographs of the smoked or ink-writing variety (Fig. 4). Should a more complex type of recorder be desired, several turns of wire around the solenoids would provide an electrical impulse which could be utilized to operate optical or inkwriting galvanometers.

DISCUSSION

Since the basic characteristics and limitations of the bubble flow meter principles have been adequately described,[4, 5, 6] the following discussion will be restricted to specific additional data pertaining to the device presented.

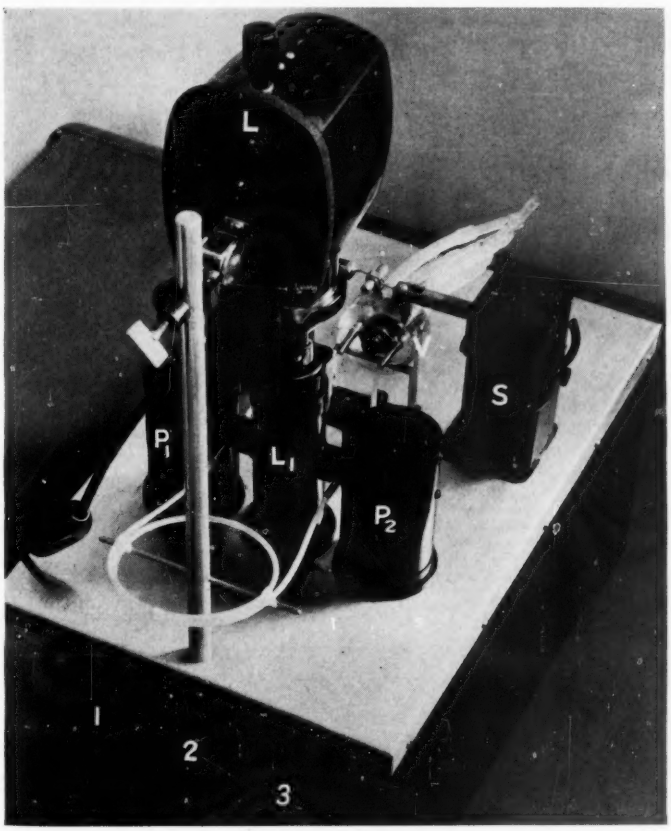

Fig. 2.—Relationships of light source, photocells, and valve-switching solenoids to valve and tube assembly. P_1 and P_2, photocell timing stations; L, light source; L_1 housing for bent Lucite tubes; S, solenoid; V, disk valve assembly; *1*, *2*, and *3*, receptacles for plugged extension cords to electronic circuit. See text for additional details.

The ratio of radius of bend (7 cm. diameter) to radius of tube (3 mm. internal bore) is 23.3. This is well within the limits set by Brunner.[4] The plastic loop T is 60 cm. long between the photocell sites. A pressure drop of 8 mm. of mercury was found to occur at a flow of 59 ml. per minute. No provision was made for maintaining constant temperature.

With proper adjustment of photocell sensitivity a 0.1 c.c. air bubble will operate the device continuously for one to two hours. As the volume of the air bubble is gradually absorbed by the blood, a small error is introduced. This error ultimately reaches the order of approximately 1 per cent at the time that a new bubble is injected. However, the removal of the human equa-

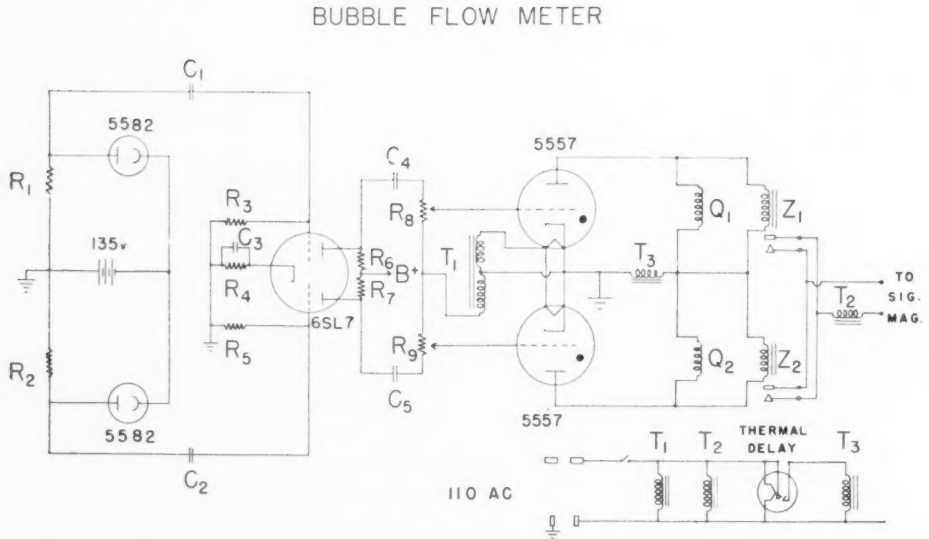

Fig. 3.—Electronic circuit controlling valve-switching solenoids and providing signal magnet recording (see text).

R_1 and R_2—0.3 Meg.
R_3 and R_5—2 Meg.
R_4—2,200 Ohms
R_6 and R_7—0.1 Meg.
R_8 and R_9—1 Meg.
C_1 and C_2—0.5 μfd.
C_3—200 μfd.
C_4 and C_5—1 μfd.

T_1—Primary 117 v; Secondaries 2.5 v, 10 v
T_2—Primary 117 v; Secondaries 2.5 v
T_3—Primary 117 v; Secondary 220 v
Q_1 and Q_2—110 v solenoids
Z_1 and Z_2—Relays

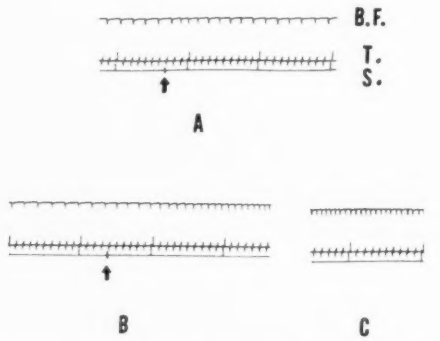

Fig. 4.—*B.F.*, Blood flow; *T*, time in 6 sec. intervals; *S*, signal; *A*, short-acting drug (papaverine) injected locally into the coronary circulation; *B*, long-acting drug (khellin) injected systemically (i.v.); and *C*, a continuation of tracing *B* at a later time interval.

tion in visual measurement of transit time offsets this small discrepancy. We have employed 0.1 c.c. of liquid petrolatum in place of the air bubble in an attempt to avoid gas absorption, but small globules were gradually dissected off the main body of the oil by the blood.

SUMMARY

A new bubble flow meter is described which employs a specially designed disk valve to retain a single air bubble and permit continuous automatic

recording. Oscillation of the bubble between photocell timing stations permits accurate measurement of the rate of blood flow for long periods of time and also operates the disk valve.

Many applications of the presented valve-switching principle are apparent in various fields of flow measurements.

We wish to express our appreciation to H. J. Cromley and H. W. Cromley for their assistance in the construction of this apparatus.

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THE USE OF THE ELECTROKYMOGRAPH FOR DETERMINATION OF PULSE WAVE VELOCITY

FREEMAN L. RAWSON, JR., M.D.
BALTIMORE, MD.

SINCE the middle of the last century there has been interest in the velocity of the pulse wave as a key to the morphology and elasticity of arteries. Although normal values in man and their variation with age, along with the changes characteristic of arterial obstruction, arteriosclerosis, and hypertension, have been elucidated, work has been hampered by lack of a readily available central pulse as a starting point. Fc this purpose various investigators have used the R wave of the electrocardiogram,[1] the apex beat,[1] the carotid pulse,[2] and the subclavian pulse.[3]

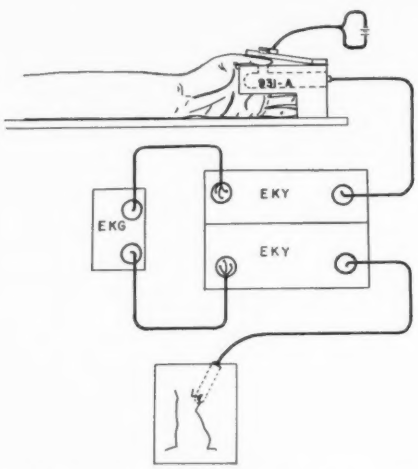

Fig. 1.—Diagram showing the photoelectric digital pulse tracer used, and its relationship to the electrokymograph.

It appears that Boone, Chamberlain, Gillick, Henny, and Oppenheimer[6] first suggested that the electrokymograph could be used for pulse wave velocity measurements and that a modification of the same instrument as a finger pulse tracer would record the arrival of the wave peripherally (Fig. 1).

We have found this to be a practical method of obtaining pulse wave velocities in the segment from aortic knob to thumb pad. A tracing of the thumb pad pulse was made by placing it between a direct current light source at the RCA 931-A photomultiplier tube. The electrokymogram was recorded simultaneously on a three string Cambridge electrocardiograph. The camera

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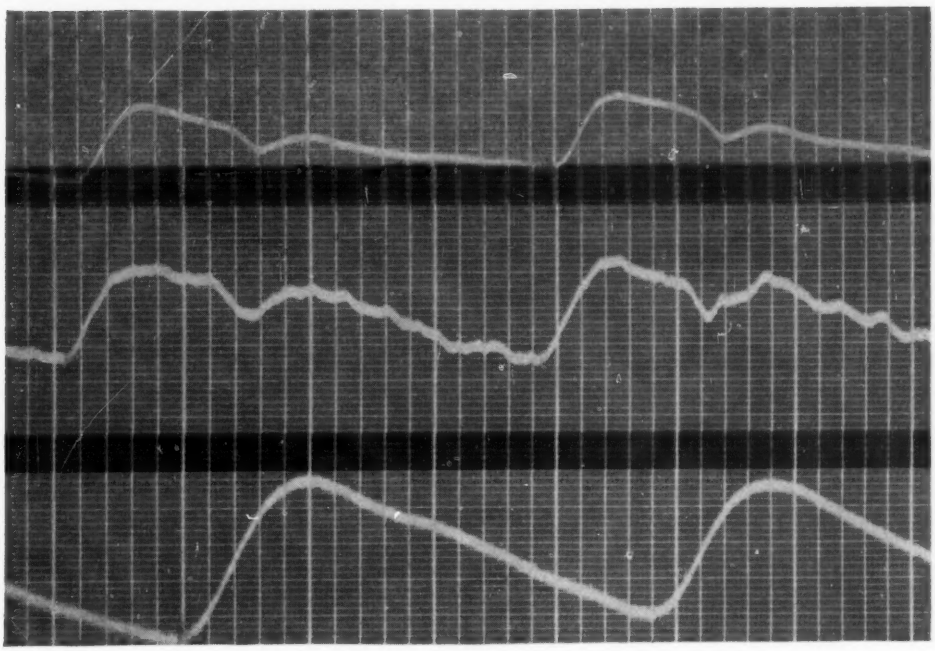

Fig. 2.—A typical tracing. Top, the carotid pulse recorded mechanically; center, the the electrokymogram; and bottom, the thumb pulse tracing. The time lines allow transmission times to be read directly.

was operated at speeds of 50 or 100 mm. per second. Heart sounds, electrocardiogram and mechanical tracings of carotid pulsations were added as desired. In order to minimize respiratory variation, voluntary apnea was maintained by the subject at the resting expiratory level (Fig. 2). Measurements of vessel length were done by a topographic technique using the second left interspace at the sternal border for the aortic knob and measuring to the thumb with the arm at a 90 degree angle with the body.

Pulse wave velocities have been generally in agreement with those reported in the past for the carotid-radial segment,[2] and have shown the reported increase in velocity with hypertension and decrease in the presence of arterial obstruction[4] (Fig. 3).

The chief advantage of this method would seem to lie in the recording of the aortic knob, in that it is easily obtained and needs no correction of vessel measurements. The aortic knob electrokymogram is, however, subject to the criticism that positional movements of the aorta may distort the initial deflection.

Piezoelectric recorders or other types without lag would provide a tracing of pulsations at intermediate points or could replace the finger pulse tracer if desired.

It is felt that modifications of this method may prove applicable to other arterial segments.

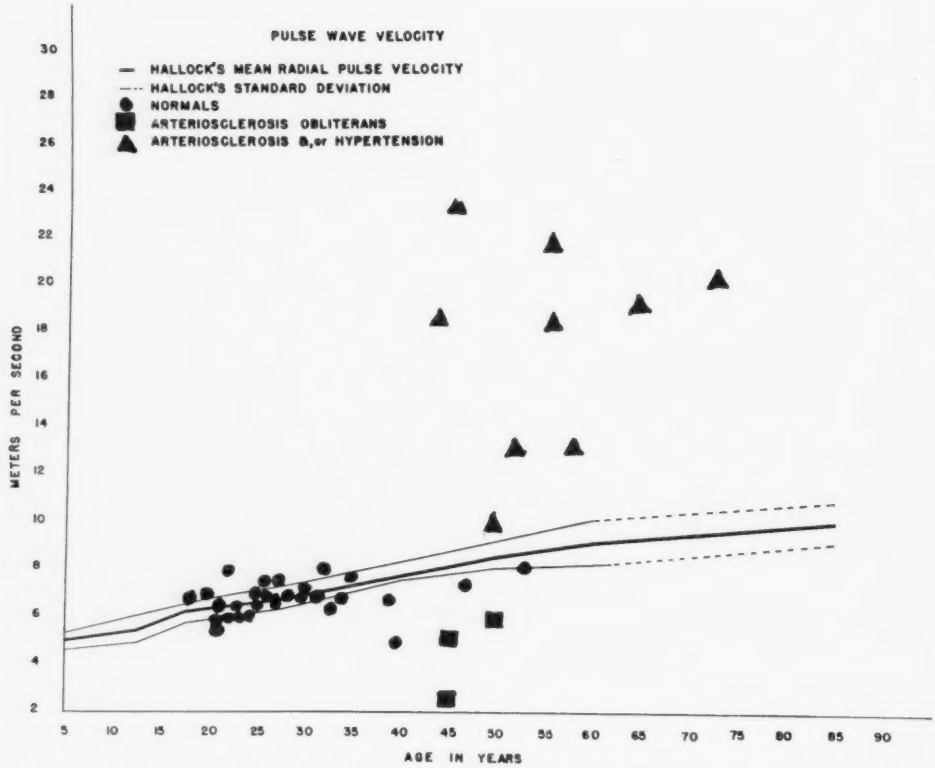

Fig. 3.—Graph showing our preliminary results plotted on the normal carotid-radial segment velocity as reported by Hallock.[2] Note the low pulse wave velocities found in limbs known to be the site of arteriosclerosis obliterans.

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